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COUPLING CONSTRUCTED WETLANDS AND ADVANCED OXIDATION PROCESSES

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À minha família e ao meu namorado

"A dúvida é o princípio da sabedoria."

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Abstract

Water pollution is one of the most serious ecological threat for humans. The presence of organic micropollutants (MPs) in the aquatic environment, usually at trace concentrations (between ng L^{-1} and µg L^{-1}), has been highlighted in the last decades as a worldwide environmental concern due to their difficult elimination by conventional water/wastewater treatment processes. Some MPs are classified as priority substances (PSs) and others as contaminants of emerging concern (CECs) depending on the respective existence or absence of regulation. MPs reach the environment through several pathways, one of them being aquaculture effluents. However, alternative technologies to eliminate organic MPs from aquaculture effluents are still poorly investigated.

In this work, a solid phase extraction coupled to ultra-high performance liquid chromatography (SPE-UHPLC-MS/MS) method ("A") was fully optimized and validated to determine 4 veterinary pharmaceuticals (ofloxacin, enrofloxacin, ceftiofur, ivermectin) in aquaculture effluents, due their frequent application in fish farms. Other SPE-UHPLC-MS/MS method ("B") was employed, after being adapted and revalidated, for analysis of MPs included: (i) in the Directive 2013/39/EU (3 pesticides and 1 industrial compound); (ii) in the Watch List of Decision 2015/495/EU (6 pesticides, 4 pharmaceuticals and 1 organic UV filter); (iii) as well as typical recalcitrant compounds in surface water (carbamazepine and clofibric acid). The optimized SPE procedure used HLB cartridges to extract 500 mL of acidified water samples and ethanol as solvent. The mobile phase optimized for method "A" consisted in a gradient of methanol and water containing 0.1% formic acid at a flow rate of 0.20 mL min^{-1}. Mass spectrometry conditions were also optimized to enable the identification and quantification of MPs, by their ion ratio and retention time, in accordance to the Decision 2002/657/EC. All compounds (analysed by methods "A" and "B") showed linearity ($r^2 > 0.99$) within the range, selectivity and sensitivity for different concentration ranges. Method detection and quantification limits were lower than 0.75 and 2.27 ng L^{-1}, respectively. Recoveries were generally higher than 85%, except for ceftiofur (20.93%), enrofloxacin (6.20%) and ofloxacin (3.67%). Accuracy varied from 81% to 116% and precision (RSD) was lower than 20%, complying the limits defined by international guidelines.

The mentioned methods were applied to analyse MPs at inlet and outlet water samples collected (March and May, 2016) in a freshwater aquaculture farm located in Portugal. While atrazine, simazine (PSs) and EHMC (CEC) were found at ng L^{-1} levels in both inlet and outlet aquaculture water samples, erythromycin was detected and quantified at ng L^{-1} levels only in the aquaculture effluents. EHMC was found at higher concentration in May, possibly due to different weather conditions and industrial discharges to the river. Then, constructed wetland (CW) systems coupled to the ozonation process were tested to remove MPs from aquaculture effluents. Non-spiked effluents containing the detected MPs (atrazine, simazine, EHMC and erythromycin) were treated, as well as spiked effluents containing the same compounds together with the 4 veterinary drugs and the 2 selected recalcitrant compounds referred above (all spiked at 100 ng L^{-1}). CW systems showed good performance to remove all target MPs with exception of EHMC; however, EHMC was efficiently eliminated in subsequent short ozonation experiments. Thus, the coupled CW-ozonation treatment was proven to be a good alternative for the removal of all MPs. Nevertheless, more bench-scale research on this subject is needed before possible application at pilot-scale.

Keywords: Priority substances; Contaminants of emerging concern; Solid Phase Extraction; Ultra-high-performance liquid chromatography; Mass spectrometry; Aquaculture effluents; Constructed Wetlands; Ozonation.

Resumo

A poluição da água é uma das mais sérias ameaças ecológicas para os seres humanos. A presença de micropoluentes (MPs) orgânicos no ambiente aquático, normalmente em concentrações vestigiais (entre ng L^{-1} e µg L^{-1}), tem sido alvo de destaque nas últimas décadas, apresentando-se como um sério problema ambiental a nível global, devido à difícil eliminação destes MPs pelos processos convencionais utilizados para o tratamento de águas e águas residuais. Estes contaminantes incluem várias classes de substâncias, nomeadamente as classificadas como prioritárias (PSs) ou os contaminantes de preocupação emergente (CECs), dependendo respetivamente da existência ou não de regulamentação. Os MPs podem chegar ao meio ambiente através de diversas vias, sendo uma delas os efluentes resultantes da aquacultura. Apesar disto, tecnologias alternativas capazes de eliminar MPs orgânicos destes efluentes, são ainda muito pouco abordadas.

Neste trabalho, um método analítico (Método "A") englobando a extração em fase sólida e cromatografia líquida de ultra alta eficiência (SPE-UHLPC-MS/MS), foi otimizado e validado, com o objetivo de determinar 4 fármacos frequentemente utilizados em práticas de aquacultura (ofloxacina, enrofloxacina, ceftiofur, ivermectina). Foi ainda aplicado um outro método SPE-UHPLC-MS/MS (Método "B"), neste caso adaptado e revalidado, para análise dos MPs incluídos: (i) na Diretiva 2013/39/EU (3 pesticidas e 1 composto industrial); (ii) na lista de vigilância da Comissão Europeia 2015/495/EU (6 pesticidas, 4 fármacos e 1 filtro UV orgânico); (iii) bem como 2 compostos recalcitrantes em aguas superficiais (carbamazepina e ácido clofíbrico). Da otimização do procedimento de SPE, resultou o uso de cartuchos HLB para extrair 500 mL de amostra de água acidificada, utilizando etanol como solvente. A fase móvel otimizada para o método "A" consistiu num gradiente de metanol e água contendo 0.1% de ácido fórmico, usando um caudal de 0.2 mL min^{-1}. As condições de espectrometria de massa foram também otimizadas, permitindo a identificação e quantificação dos MPs de acordo com a Decisão 2002/657/EC. Todos os compostos (analisados pelos métodos "A" e "B") apresentaram linearidade ($r^2 > 0.99$), seletividade e sensibilidade nas respetivas gamas de concentrações definidas. Valores menores ou iguais a 0.75 e 2.27 ng L^{-1}, foram obtidos para os limites de deteção e quantificação dos métodos, respetivamente. Registaram-se recuperações maiores do que 85% para a maioria dos poluentes, exceto para ceftiofur (20.93%), enrofloxacina (6.20%) e ofloxacina (3.67%). A exatidão variou entre 81% e 116% e a precisão (desvio padrão relativo) foi inferior a 20%, cumprindo os limites definidos pelas diretrizes internacionais.

Os métodos descritos foram aplicados para analisar MPs em amostras recolhidas à entrada e saída (em Março e Maio 2016) de uma exploração de aquacultura de água doce, localizada em Portugal. Enquanto os PSs atrazina e simazina e o CEC 4-metoxicinamato de 2-etil-hexilo (EHMC), foram encontradas tanto nas amostras da água de entrada como nas de saída, em concentrações na ordem dos ng L^{-1}, a eritromicina foi detetada e quantificada apenas nas águas de saída, na mesma gama de concentrações. O EHMC foi determinado em concentrações mais elevadas em Maio, possivelmente devido às diferenças verificadas nas condições atmosféricas e/ou descargas industriais efetuadas no rio. Foi também estudada a viabilidade de um sistema de tratamento biológico (leito de macrófitas / "constructed wetlands" - CW) acoplado a um processo de ozonização para remoção dos MPs encontrados nos efluentes de aquacultura (atrazina, simazina, EHMC e eritromicina). Foram também realizados ensaios utilizando a mesma matriz, após contaminação com estes 4 compostos, 4 fármacos veterinários e 2 compostos recalcitrantes acima identificados (100 ng L^{-1} de cada MP). O sistema biológico CW demonstrou uma boa eficiência na remoção de todos os compostos em análise, com a exceção do EHMC; contudo, este foi

eficientemente eliminado no processo de ozonização em ensaios de curta duração. O tratamento acoplado demonstrou ser uma boa alternativa para remover os MPs em estudo. No entanto, é necessária mais pesquisa à escala laboratorial, antes da possível aplicação à escala piloto.

Palavras-chave: Substâncias prioritárias; Contaminantes de preocupação emergente; Extração em fase sólida; Cromatografia líquida de ultra alta eficiência; Espectrometria de massa; Efluentes de aquacultura; Leito de macrófitas; Ozonização.

Nomenclature

AC - Activated carbon

AOP - Advanced oxidation process

BOD - Biochemical oxygen demand

CE - Collision energy

CEC - Contaminant of emerging concern

CW - Constructed wetland

DOC - Dissolved organic carbon

DP - Declustering potential

DWTP - Drinking water treatment plant

EC - European Commission

EDC - Endocrine disrupting compound

EDTA - Ethylenediaminetetraacetic acid

E1 – Estrone

E2 - Estradiol

EE2 - Ethinylestradiol

EHMC - 2-Ethylhexyl 4-methoxycinnamate

EQS - Environmental quality standards

ESI - Electrospray ionization

EU - European Union

GC - Gas chromatography

HLB - Hydrophilic–Lipophilic–Balanced

HRT - Hydraulic residence time

HSSF-CW - Horizontal subsurface flow constructed wetland

IDL - Instrument detection limit

IQL - Instrument quantification limit

LC - Liquid chromatography

LLE - Liquid–liquid extraction

MAX - Mixed–mode anion eXchange

MBR - Membrane bioreactors

MCX - Mixed–mode cation eXchange

MDL - Method detection limit

ME - Matrix effect

MP - Micropollutant

MQL - Method quantification limit

MS – Mass spectrometry

MS/MS - Tandem mass spectrometry

Mw - Molecular weight

PFOS - Perfluorooctanesulfonic acid

PPCPs - Pharmaceuticals and personal care products

POP - Persistent organic pollutant

PS - Priority substance

QC - Quality control

QqQ - Triple quadrupole

RSD - Relative standard deviation

SPE - Solid phase extraction

SPME - Solid phase micro extraction

SF-CW - Surface free water constructed wetlands

SPME - Solid phase micro extraction

SRM - Selected reaction monitoring

SST - Total suspended solids

UHPLC - Ultra–high-performance liquid chromatography

VSSF-CWs - Vertical subsurface flow constructed wetlands

WFD - Water framework directive

WWTP - Wastewater treatment plant

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Introduction

1.1. Overview of the problem

Water is essential for life, and undoubtedly the most important resource on Earth. However, it is likely to become a more critical and scarce resource in the next decades. Water covers approximately 70 percent of the Earth's surface, from which 97 percent is salty water (oceans and seas) and only the remaining 3 percent is fresh water, distributed by ice sheets, aquifers, lakes, rivers, ponds, and atmosphere. Nonetheless, only 0.002 percent of this fresh water stock is available for human use, hence it is essential to preserve its quality [1].

The term "water quality" is closely related to water pollution and is among the most serious ecological threats that humans face today. It is important to perceive that meeting global water quality is a requirement for sustainable development of the Earth and overall well-being of the present and future generations. Water quality is intrinsically related to human health, food safety, poverty reduction, preservation of ecosystems and other factors, such as economic growth and social development of our societies [2]. The continuous growth of population and urbanisation led to an increase of water usage for domestic, agricultural and industrial purposes and resulted in a great production of wastewater, directly released into the environment *(Figure 1)* [1]. Thus, water pollution represents a challenge to overcome, not only to ensure good quality water for human needs, but also to satisfy ecosystem requirements [2].

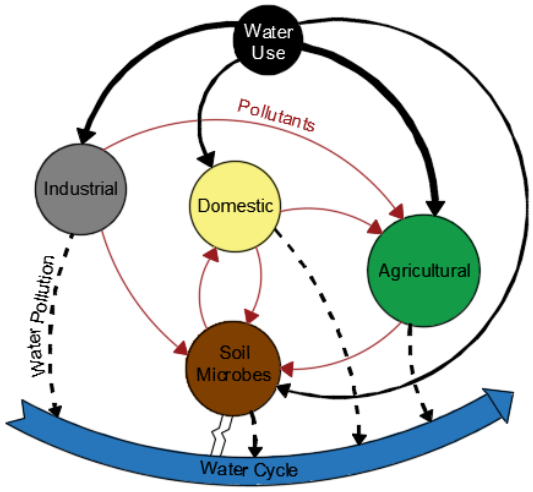


Figure 1. Sources of water quality deterioration (Adapted from ref. [1]).

Water pollution can be defined by several ways, nevertheless, the basic elements of most definitions, are the presence of particular pollutants at certain concentrations and during a period of time enough to cause undesirable effects [1].

Until very recently, the main focus on the impacts of water pollution has been related to conventional pollutants, such as metals and persistent organic pollutants (POPs). For this reason, they were extensively studied, being developed measures to avoid and/or control their presence in water [3]. However, over the last few decades, the occurrence of micropollutants (MPs) in the aquatic environment has become a worldwide issue of increasing environmental concern [4]. MPs can be anthropogenic or natural substances, commonly present in water at trace concentrations, ranging from a few ng L^{-1} to several µg L^{-1}. These compounds include classes such as pharmaceuticals, personal care products, steroid hormones, industrial chemicals and pesticides, integrated on the so-called priority and emerging contaminants [4]. Industrial and domestic wastewaters, hospital effluents, landfill leachates, runoff from agriculture, livestock and aquaculture, are the main sources responsible for their continuous and uncontrolled introduction into the aquatic systems. Despite some of them persist in the environment, others are considered "pseudo-persistent" due to their continuous introduction at low levels into the environment exceeding their transformation or removal rate [5]. The "low concentration" and diversity of MPs, complicate not only the associated analytical procedures for their detection, but also create challenges for conventional water and wastewater treatment processes. These systems are not specifically designated to eliminate MPs, thus, many of these compounds remain in the aquatic environment, leading to adverse effects for wildlife and human health [4].

1.2. Priority substances and Emerging contaminants

MPs are classified as priority and emerging contaminants, depending on whether exist or not legislation, respectively. PSs are defined, in the Article 16 of Water Framework Directive (WFD) 2000/60/EC, as *"individual pollutants or groups of pollutants presenting a significant risk to or via the aquatic environment, including such risks to waters used for the abstraction of drinking water"* [6]. Most of them are organic contaminants, but some toxic metals and organometallic compounds are also included [7]. Emerging contaminants (ECs) or contaminants of emerging concern (CECs) are the terms that have been used to identify chemicals and microbial constituents that are not regulated yet. CECs include substances that have been recently detected in natural streams (often due to improved analytical detection capacity) and/or pose risk to human health and/or ecosystems, which are not fully understood yet. CECs are not necessarily new substances, they include pollutants that have been present in the environment over the years, but which presence and importance are only now recognized and/or evaluated [8]. CECs include several types of products such as pharmaceuticals and personal care products (PPCPs), steroids and hormones, pesticides, industrial and household chemicals, metals, surfactants, industrial additives and solvents, among others.

The European WFD 2000/60/EC Directive [6] is probably the most significant international legislation introduced in the field of water for many years. Its primary aim is the achievement of "good status" in all water bodies, and therefore, its implementation intends to intensify the monitoring of ecosystems and enhance the control of contaminants. The referred Directive sets out the European Union (EU) strategy against pollution of water by chemical compounds, identifying PSs with high risk to the aquatic systems *(Article 16)* [7]. Later, in 2008, a list of 33 PSs was established by the Directive 2008/105/EC [9], enforcing the European Commission (EC) to review it at least every four years. Environmental quality standards (EQS) were also defined for these groups of substances and other 8 pollutants were

highlighted, based on available data of acute and chronic effects to aquatic environment and human health [5].

Recently, on August 2013, the Directive 2013/39/EU was published amending the previous Directives 2000/60/EC and 2008/19/EC [10], and updated the water framework policy. This Directive suggests several changes, namely related with PSs:

i) new PSs were identified;
ii) EQS for the newly identified substances were defined, which should be met by the end of 2027;
iii) EQS for substances already identified were revised, which should be met by the end of 2021;
iv) biota EQS were defined for some existing and newly identified PSs.

Directive 2013/39/EU includes a list of 45 PSs and also certain other pollutants with EQS to be considered *(*Appendix A1*)*. It reinforces the preventive action (highlighting the polluter pays principle), the identification of pollution causes, the need to deal with emissions of pollutants at the source and to develop innovative water/wastewater treatment technologies [5].

It is important to stress that Directive 2013/39/EU calls the attention to the significance of monitoring the CECs for which legislation do not exist. In this context, EC established a list of 10 substances/groups of substances in a entitled Watch List [11] (Appendix A2), in order to be monitored in surface water within the EU [5]. Thus, the concern of the EC about these substances is clear.

1.3. MPs in the aquatic systems

1.3.1. Pollution sources

MPs reach the environment through diverse sources, such as industrial, domestic, hospital, agriculture/livestock and aquaculture. *Figure 2* shows some possible pathways of these contaminants in the aquatic systems.

Typically, MPs are synthetic compounds generated via human activity (anthropogenic origin). Industry is responsible for production of several compounds, such as pesticides, PPCP, among many others common in the daily use. It encompasses the steps of manufacturing, processing and distribution by the various sectors (health, domestic, agricultural, etc.) [12].

As result, effluents containing various contaminants including MPs are produced, being one of the possible sources responsible for their presence in the environment. Nevertheless, since the industrial effluents should be properly treated and/or routed to municipal wastewater treatment plants (WWTPs), industry is not considered the major “threat” to water courses.

Hospital effluents and domestic wastewater are other possible sources of MPs. Usually, pharmaceuticals are only partly metabolised in the human body, and therefore, the remaining fractions are excreted via urine and faeces, reaching the WWTPs through their effluents [12]. In addition, other products containing MPs (e.g. soaps, shampoo, toothpaste, disinfectants, etc.) are commonly used in houses and hospitals, following the same destination.

Agriculture is another important source of MPs, mainly for pesticides that are used to improve the productivity (e.g. controlling the pests and vectors). After heavy rainfall, a variety of pesticides can be found in waterways, as result of agricultural runoff. Veterinary drugs and food additives used for livestock (excreted in urine and faeces), also may enter in the aquatic systems via runoff. In addition, a

fraction of these pollutants can infiltrate the soil and pollute the groundwater system. Leaching from dumping sites and sewage treatment facilities, as well as environmental disasters, are other sources of MPs [12].

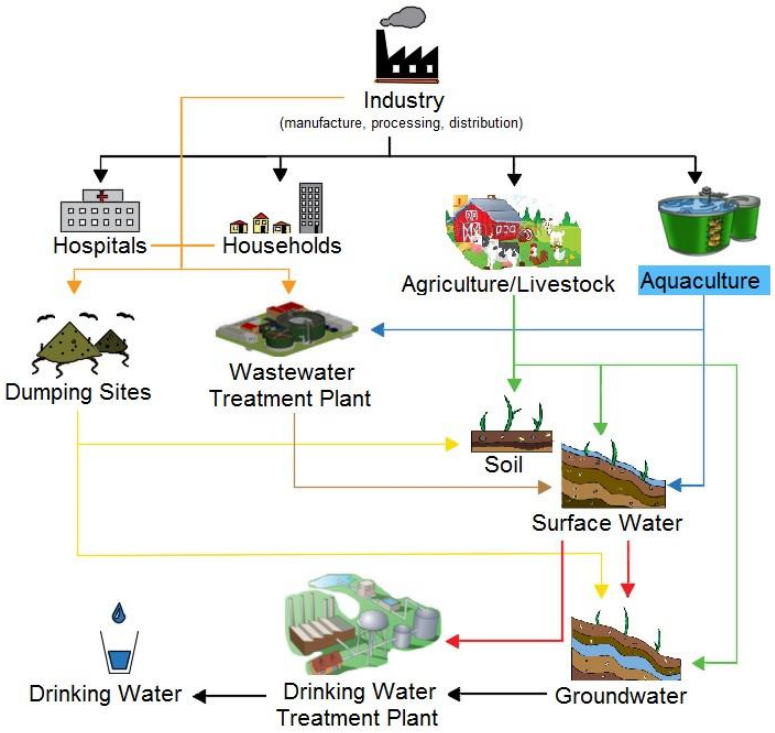


Figure 2. Pathways of MPs into the environment (Adapted from ref. [12]).

Despite the importance of the sources mentioned, it is consensual that the most significant source of MPs in the aquatic systems is the discharge of treated effluents from domestic WWTPs [12]. Although many substances (e.g. particulates, nutrients and pathogens) are efficiently eliminated, the removal of MPs is often insufficient. Conventional WWTPs are not specifically designed to eliminate MPs and therefore, many of these compounds are able to pass thought wastewater treatment processes by virtue of their persistence and/or their continuous introduction at residual levels. Consequently, introduction into the receiving surface water constitutes a threat not only to wildlife, but also to human health [4]. Once surface water is widely used as raw water to produce drinking water and the drinking water treatment plants (DWTPs) are not able for their complete removal, tap water containing MPs can be ingested by humans, exposing them to such substances and their effects. In addition, surface water is used for many activities in food industry, including water collected from rivers for aquaculture.

1.3.2. Environmental fate and effects of MPs

The environmental fate of MPs is basically determined by two factors: **(i)** the properties of the compound itself, and **(ii)** the conditions of the surrounding system. Depending of these, processes such as sorption, volatilization, dispersion, hydrolysis, oxidation, isomerisation and photodegradation can occur [12]. As the environmental compartments are constantly interconnected, the MPs are spread by surface water,

groundwater, soil and air. Furthermore, they may also bioaccumulate in plants and other organisms (humans or animal) [13]. *Figure 3* presents the fate of MPs in water systems.

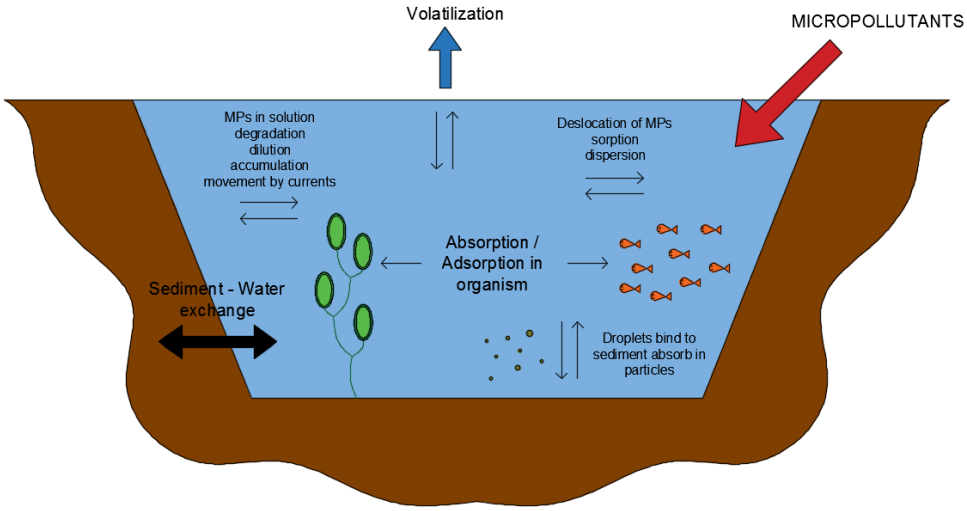


Figure 3. Fate of MPs in water systems (Adapted from ref. [12]).

The occurrence of MPs in the aquatic environment has been frequently associated to negatives effects to the ecossystem and human health [14]. However, these effects are still uncompletely understood due to the variability obtained in different studies. Some reports have shown that exposure to certain MPs even at very low concentrations can originate impacts on biological systems, mostly in several fish and aquatic species [15].

Bacterial resistance represents a serious threat to human health, since infections known to be easily controlled with available treatments can re-emerge as significant public health problems. Antibiotics and disinfectants are the most common antimicrobial agents able to contribute to the increase of antimicrobial resistance. Recently, some researchers found higher levels of antibiotic resistance genes downstream of a WWTPs compared to upstream, suggesting that WWTPs effluents can be a significant source for the spread of antibiotic resistance in the environment [15]. Concerning acute toxic effects, the concentrations of MPs in water are quite low, so acute toxicity is unlikely to occur, but the precautionary principle should be kept in mind due to the possible chronic and long-term exposure that are more difficult to evaluate [14].

Besides the resistance mechanims developed by bacteria and other toxic effects, another high concern is related to endocrine disruption, i.e., the interference of MPs with the proper function of the endocrine system, which is responsible for controling important body functions such as growth, metabolism and reproduction. Endocrine disrupting compounds (EDCs) such as personal care products, pesticides, hormones, steroids and solvents, are known to cause tumors, birth deffects and development disorders [15]. EDCs may interfere with organisms endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife [2]. Several studies have also reported low sperm count, reduced fertility and reproductive malfunctions in aquatic species exposed to EDCs [15]. While adverse effects on fish and other species have been demonstrated, the effects on human health is still a subject of discussion which needs more investigation [14]. It is important to mention that according to some authors, DWTPs might additionally increase the endocrine disrupting of treated water through the chlorination step, by generating chlorinated by-products [13].

1.3.3. Aquaculture: A case study of food industry

Aquaculture is the farming (in the fresh, salty or brackish water) of aquatic organisms including fish, molluscs, crustaceans and aquatic plants, using techniques developed to increase their production beyond the natural capacity of the environment. It is classified as extensive, semi-intensive and intensive, according to control of production levels and need for furnishing feed and food supplements, respectively, almost nil, media and high. The intensive aquaculture is normally associated to the production of aquatic organisms in large quantities (industry scale), involving the control of breeding and the supply of artificial feed and medication. Its recent growth has generated high concern at environment level and their sustainability has been questioned, due to its potential environmental impacts and consequently risk for the human health [16].

It is important to underline that recent estimations show that aquaculture provides 47% of global fish consumption. In order to keep up with population growth and increasing *per capita* fish consumption and an increase of 60-100% over the next 20-30 years is expected [17]. These facts only stress the attention that should be given to the environmental pollution caused by this type of food industry.

Aquaculture effluents can contain various types of substances. Because of excess feed supply and excrements of the cultured organisms, the most common are dissolved inorganic nutrients, such as nitrogen (N) and phosphorus (P) that can cause hypertrophication when released into receiving environment. These nutrients, together with organic solids, have been the focus of most studies related with impact of the aquaculture effluents [18].Other chemicals including MPs such as disinfectants, antifoulants, estrogens and veterinary drugs, are applied in aquaculture in order to increase efficiency, improve survival rates and control pathogens and diseases. The dispersion of these compounds in the water bodies is other important concern on the pollution caused by such practices. For this reason, the environmental assessment in the aquatic systems of chemicals present in the aquaculture effluents, namely MPs, requires more investigation, even because little is known about their possible consequences [16].

1.4. Analytical methods for determination of MPs in water

The determination of MPs is fundamental to assess water contamination, being one of the most important fields of modern analytical chemistry. The analytical methods used for this purpose, should be selective and specific, sensitive and accurate [19] involving not only the sample analysis, but also the sample preparation, which is a labour-intensive step *(Figure 4)* [20]. However, and given its complexity, there are still no standardized methods available for MPs analysis [20].

There are multiple sample preparation techniques that can be applied to water samples, depending on the analytes and the matrix. The aims of these techniques are: (i) to remove potential interferences from the sample matrix; (ii) to increase the concentration of target analytes, allowing their detection; (iii) to provide a robust and reproducible method; and (iv) if necessary, to convert the analytes into a more suitable form (e.g., via derivatization or pH adjustment). Pre-treatment, clean-up and concentration are the main steps included in most sample preparation methods [20].

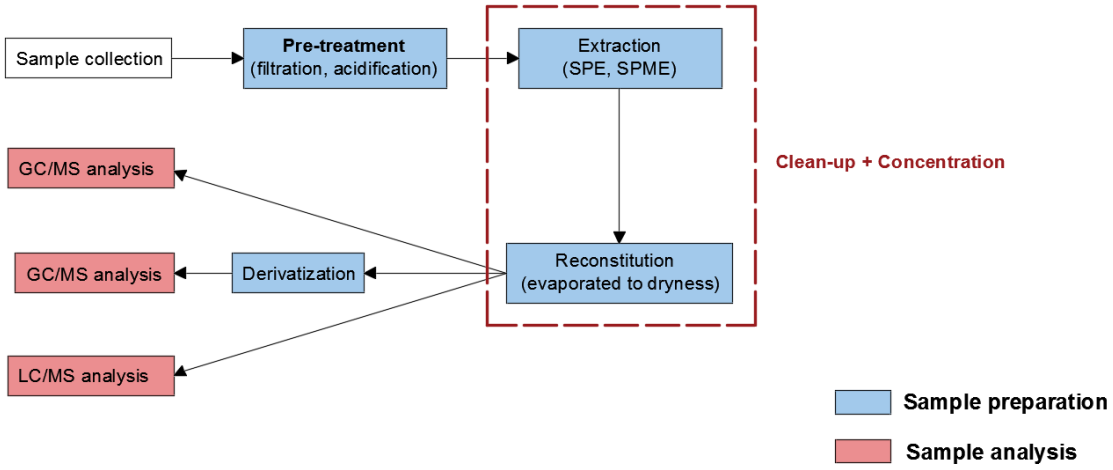


Figure 4. Overview of the main sample preparation and analysis steps (Adapted from ref. [20]).

Pre-treatment, generally includes filtration and/or pH adjustment of the environmental samples. Thus, the presence of interfering species and clogging risk during extraction are minimized and the chemical properties of the analytes are maintained, favouring their stability and interaction with the sorbent or solvent in the extraction step. Sample clean-up is usually performed by solid-phase extraction (SPE) and involves conditioning the sorbent, percolating the sample, rising and cleaning the sorbent, eluting the sample and analytes recovery [20]. Besides SPE, other techniques can be used to clean-up water samples. Liquid-liquid extraction (LLE) is a conventional method with several shortcomings, such as time-consuming and large volumes of (toxic) organic solvent needed, and therefore it has been replaced by SPE in recent years [21]. Solid phase microextraction (SPME) is a more recent technique applied and surpass some limitations inherent to SPE and LLE [22]. To be used in LC, it needs to be coupled to a desorption chamber for solvent desorption before LC or to use an open tubular fused-silica capillary column instead of a SPME fiber [23]. The principle of SPE is simple and consists in the use of sorbent and solvents. The sorbent is a stationary phase normally packed in a cartridge or alternatively immobilized in a membrane and the mobile phase consists of an organic solvent or a mixture of aqueous and organic solvents, during the elution step. High recoveries of MPs are obtained when the analytes are strongly retained by the sorbent in presence of water, and have a subsequent higher affinity to the eluent during elution phase. The efficiency of extraction is affected by the type of sorbent, and also by other parameters, such as the solvent(s) used, sample pH, sample volume, among others. These parameters might be carefully optimized in order to obtain high recoveries and low interferences. After the elution step, the sample extract is further concentrated by evaporation and reconstituted with a known volume of an appropriate solvent [20]. The process of sample concentration is very important since there are very few analytical methods that can be used for the determination of MPs in their original concentration [19].

The sample analysis require highly sensitive methods that enable detection and quantification of organic MPs. Liquid chromatography (LC) is typically used to determine more polar and less volatile compounds, while gas chromatography (GC) is used for less polar volatile and semi-volatile compounds. It is possible to analyse polar compounds by GC, however it requires an additional step of derivatization, which allows to increase their volatility and thermal stability [20]. For environmental analyses, LC or GC are normally coupled to tandem mass spectrometry (LC-MS/MS; GC-MS/MS) [21]. Nevertheless, in the specific case of this work, the attention is focussed on the LC-MS/MS methodology. LC is a

powerful and versatile separation technique and MS is a sensitive technique of detection and identification [21]. In LC, the compounds are forced to pass through a column (stationary phase) by a solvent and separated by different chemical interactions between the analytes and the stationary phase. Recently, ultra-high performance liquid chromatography (UHPLC) is preferred over conventional LC, since it offers improvements in speed, resolution and sensitivity [24]. Tandem MS is an identification technique where molecules are charged into ions, which are then fragmented and the respective produced fragments are analysed, on the basis of their mass to charge ratio (m/z) [25]. The ion sources that can be coupled to LC and used to ionize the analytes are electrospray ionization (ESI) and atmospheric pressure chemical (APCI), both using atmospheric pressure ionization (API). Both produce protonated or deprotonated molecules and others, being ESI more suitable for analysis of polar compounds and APCI for analysis of medium to low polar substances. There are various analysers for MS detectors, which are important to separate ions according to their m/z and to fragment them, applying electromagnetic field. Triple quadrupole (QqQ) is the most used for environmental analysis. Thus, currently UHPLC-MS/MS is one of the most advanced analytical technology for determination of MPs in water. However, the matrix effect in the ionization source is the main drawback and may result in the suppression or, less frequently in the enhancement of analyte signals. These matrix effects are important to be considered in order to avoid an erroneous interpretation of results. Optimizing LC and MS parameters is therefore fundamental to obtain reliable results [20].

1.5. Treatments for removal of MPs in water

Conventional WWTPs are not specifically designed to remove various MPs and, thus, alternative technologies are urgently required, a few have been developed at full-scale and more at bench-scale [26]. Technologies including adsorption by activated carbon (AC), some advanced oxidation processes (AOPs), such as ozonation, membrane processes and membrane bioreactors (MBR) are some examples at full-scale [4]. Most of the processes at bench-scale are not widely employed due to their expensiveness in large-scale, emphasizing the need for alternative processes with high removal efficiencies at reasonable costs [26]. In this sense, constructed wetlands (CWs) have been proved as a promising alternative due to their unique advantages of low-cost, simple operation/maintenance and environmental friendliness; however, there is a huge potential to exploit in this field of research. CWs have been demonstrated able to efficiently eliminate various contaminants, including total suspended solids (TSS), biochemical oxygen demand (BOD), nitrogen, phosphorus and metals, nevertheless the applicability for the elimination of MPs has been only recently investigated in some studies [26]. Thus, the feasibility of CWs to remove MPs from different types of wastewaters, namely aquaculture effluents, requires more research to better understand the removal of MPs and mechanisms, the influence of design and environmental factors, as well as the toxicity risks [27]. Combining or integrating CWs with other existent technologies, such as ozonation, can be also a good option, once it can maximize their individual advantages for the elimination of MPs [28], i.e. the low cost of CWs and the high efficiency (but higher cost) of ozonation. But this is still an underexplored subject.

1.5.1. Constructed wetlands (CWs)

Natural wetland systems are characterised by their capacity to remove pollutants present in water that flows through. Since these systems can improve water quality, artificial wetlands have been constructed to replicate the process [29]. Thus, CWs are currently used to treat wastewater, being most commonly applied to the treatment of secondary domestic sewage effluents or as a tertiary step [30].

CWs are complex systems containing water, substrate, plants and native microorganisms [30], where physical, chemical and biological processes may occur simultaneously (e.g., volatilization, sorption and sedimentation, photodegradation, plant uptake and microbial degradation) contributing to eliminate several compounds [26]. The general removal mechanisms occurring to eliminate MPs in CWs are illustrated in *Figure 5*.

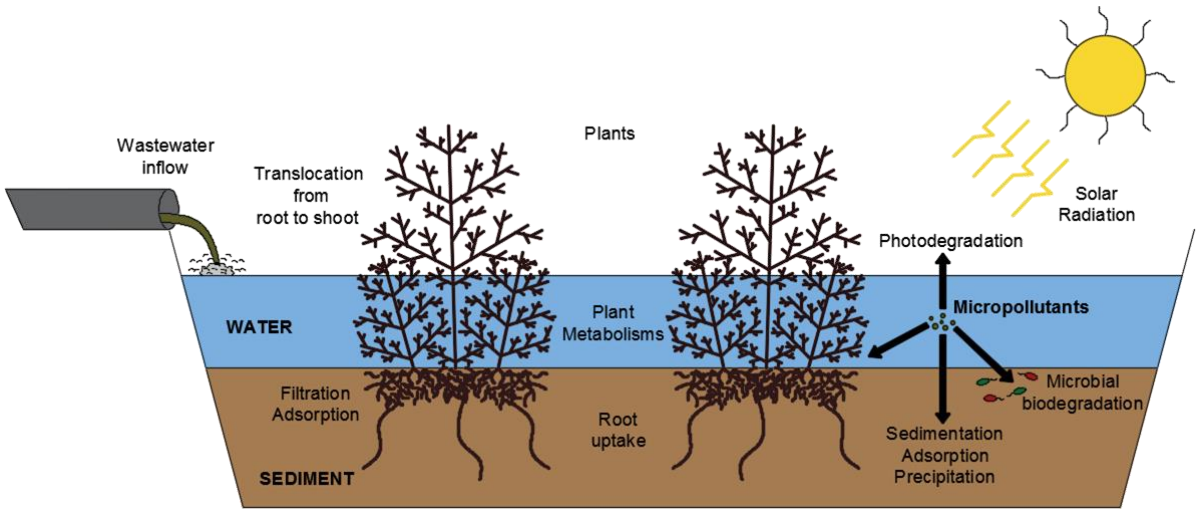


Figure 5. MPs removal mechanisms in CWs (Adapted from ref. [26])

It is important to mention that the processes aforementioned depend on a variety of design and operational factors interfering with elimination of MPs [26], such as operating mode (batch or continuous), soil matrix/substrate, depth of bed, plant species, hydraulic residence time (HRT) and wetland configuration. Relatively to the operating mode, batch (as opposed to continuous feeding) generally provides superior treatment performances, not only for conventional pollutants, but also for many MPs. This fact may be explained by the entrainment of air into the micropores of the soil matrix between the draining and flooding phases in batch operation, which promotes a micro-aerobic environment, increasing microbiological activity and consequently the degradation of organic MPs [26]. The soil matrix is important because it supports the growth of plants and microorganisms, promoting a series of chemical and physical processes. Thus, the sorption by the solid matrix can play an important role in contaminant retention, being the selection of a material with high sorption capacity essential to enhance the CWs performance. In addition, the depth of the solid matrix bed is an important design parameter already described in some works, showing that removal rates depend on the depth of solid matrix [26]. The selection of plants species is other fundamental parameter for the operation of these systems, since they have a variable capacity for nutrient uptake and soil oxygenation, also affecting the functioning and structure of bacterial communities involved in the removal of contaminants [26]. The most popular species existing in CWs are *Typha* ssp. and *Phragmites* ssp. [27]. In turn, the HRT also affects the contaminants removal efficiencies. Higher HRT allows a longer interaction of contaminants with the wetland system, while lower HRT reduces the contact time between the wastewater, the rhizosphere and the microorganisms [26]. HRT is variable and depends on the type of CWs application, however it has been reported that the most efficient pollutant removal in CW systems can be achieved in the range of 4-15 d [26]. Finally, the CWs configuration strongly influences the physical-chemical parameters (e.g. redox potential and insolation) of the system [26]. CWs can be classified according to: (i) hydrology (water surface flow and subsurface flow), (ii) plants growth form (emergent, submerged, free-floating, and floating leaved) and; (iii) flow path (horizontal and vertical). Thus, they are usually

distinguished in surface free water constructed wetland (SF-CWs), horizontal subsurface flow constructed wetlands (HSSF-CWs) and vertical subsurface flow constructed wetlands (VSSF-CWs). In addition, various types of CWs may be combined to achieve higher removal efficiency, originating hybrid systems [31]. Thereby, the configuration should be defined according to the characteristics of the contaminants to be removed. For example, SF-CWs show better performance for compounds which are susceptible to photodegradation since water is directly exposed to sunlight, while VSSF/HSSF-CWs have a higher potential to eliminate biodegradable compounds, where VSSF-CWs have an enhanced microbial degradation as result of a higher oxygenation [26].

Despite the recognised importance of the design and operational parameters, most studies dealing with MPs have been conducted only to evaluate the removal efficiencies of some specific MPs. In addition, CWs are commonly viewed as a "black box" where only influent and effluent concentrations are measured to evaluate their performance, without details about the fate or transformation pathways of the contaminants. In this regard, it is imperative to understand the basic elimination and transformation processes that drive the removal of MPs in such "black box", in order to optimize the CWs design and consequently get better treatment efficiency [26]. Removal of MPs by CWs is still a recent area of study, which needs more research to get the feasibility in terms of large-scale application. Furthermore, studies related to the application of CWs for the removal of several MPs in aquaculture, such as those referred in Directive 39/2013, are still missing.

1.5.2. Ozonation

Due the refractory nature of some MPs, several types of water treatment processes, including CWs, are still not able to provide their adequate elimination. To overcome this problem, coupling advanced oxidation processes (AOPs) such as ozonation, can be considered [4].

Ozone is a powerful oxidant which can degrade contaminants by two different pathways: **(i)** directly, via reactions with O_3, and **(ii)** indirectly, via reactions with hydroxyl radicals ($HO^{\bullet}$) mainly in alkaline conditions [5]. Whereas various pollutants are susceptible to both species (O_3 and $HO^{\bullet}$), others are only reactive towards $HO^{\bullet}$, which are strong radicals less selective than O_3 [4].

Ozonation is a promising technique to decrease considerably the concentrations of MPs in wastewaters, although it is still an expensive technology. Coupling ozonation to biological treatment processes such as CWs, can turn ozonation into a more economical treatment, because CWs act as pre-treatment, eliminating some MPs and decreasing the concentration of others [4].

One still need to be aware that the main shortcoming of ozonation is that a complete mineralization of organic compounds is not usually achieved [5]. Thus, the major concern of applying this technology is the formation of transformation products from MPs. These oxidation by-products might have toxicological effects compared to, or even higher, than the parent compounds, being important to determine and eliminate them by adequate approaches [4].

1.6. Objectives

The main aim of this work was to analyse the ability of coupling CWs and ozonation to remove a group of MPs in aquaculture effluents. Thus, the specific objectives proposed were:

- To perform a literature review on efficiency of CWs to remove MPs defined in the Directive 2013/39/EU and in the Watch List of Commission Decision 2015/495/EU;
- To develop, optimise and validate a SPE-UHPLC-MS/MS method to analyse MPs in aquaculture effluents, before and after treatment;
- To study bench-scale CW systems to remove MPs;
- To apply the ozonation process for the removal of the most recalcitrant compounds;
- To conclude about feasibility of coupling CWs and ozonation processes to remove MPs.

2
Literature review

The occurrence of MPs in aquatic systems is nowadays a recognized worldwide environmental issue. In this sense, several alternatives have been studied to overcome the difficulties related to their elimination. CWs treatments are based on aquatic plant systems that raised particular interest among the scientific community. Considering their importance in this work, a literature review is herein performed in order to gather relevant information on the applicability of CW for the removal of MPs from effluents. The survey was focused on the application of CWs for the treatment of effluents containing organic PSs defined in the Directive 2013/39/EU and CECs of the Watch List of Commission Decision 2015/495/EU. The search was done in *Scopus* database, using "constructed wetland" and the name of each MP as keywords, namely:

- "octylphenol" [32, 33];
- "nonylphenol" [32-43];
- "perfluorooctane sulfonic acid" [44-46];
- "di(2-ethylhexyl)phthalate" [47-50];
- "trichloromethane" [51];
- "dichloromethane" [51];
- "1,2-dichloroethane" [52, 53];
- "pentachlorobenzene" [54];
- "benzene" [55-79];
- "polychlorinated dibenzo-p-dioxins" [80];
- "naphthalene" [81];
- "fluoranthene" [82];
- "alachlor" [54];
- "diuron" [54, 83-85];
- "tributyltin compounds" [86];
- "simazine" [54, 85, 87-89];
- "atrazine" [85, 90-106]
- "chlorpyrifos (chlorpyrifos-ethyl)" [107-115];

- "chlorfenvinphos" [116];
- "dichlorodiphenyltrichloroethane or (DDT)" [117];
- "hexachlorobenzene" [118-121];
- "pentachlorophenol" [54, 122];
- "endosulfan" [54, 123-125];
- "dieldrin" [126];
- "imidacloprid" [127];
- "erythromycin" [128-132];
- "clarithromycin" [129-132];
- "azithromycin" [129];
- "diclofenac" [42, 128, 130, 133-154];
- "estrone" [35, 155-160];
- "17-beta-estradiol" [33, 35, 155, 157-162];
- "17-alpha-ethinylestradiol" [33, 139, 158, 159, 163].

No references were found for the other PSs and CECs, namely: "trichlorobenzenes", "trichloro-ethylene", "tetrachloro-ethylene", "carbon tetrachloride", , "chloroalkanes", "dioxin-like polychlorinated biphenyls", "polychlorinated dibenzofurans", "anthracene", "indeno(1,2,3-cd)pyrene", "benzo(k)fluoranthene", "benzo(g,h,i)perylene", "benzo(k)fluoranthene", "benzo(b)fluoranthene", "benzo(a)pyrene", "γ-hexabromocyclododecane", "β-hexabromocyclododecane", "α-hexabromocyclododecane", "1,2,5,6,9,10-hexabromocyclododecane", "1,3,5,7,9,11-hexabromocyclododecane", "heptabromodiphenylether", "hexabromodiphenylether", "pentabromodiphenylether", "tetrabromodiphenylether", "bifenox", "aclonifen", "quinoxyfen", "cypermethrin", "terbutryn", "cybutryne", "dichlorvos", "hexachlorobutadiene", "hexachlorocyclohexane", "heptachlor", "dicofol", "endrin", "isodrin", "aldrin", "triallate", "oxadiazon", "acetamiprid", "clothianidin", "thiamethoxam", "thiacloprid", "methiocarb", "2-ethylhexyl-4-methoxycinnamate" and "2,6-di-tert-butyl-4-methylphenol".

2.1. Studies dealing with the removal of PSs by CWs

The number of papers published so far about removal by CWs of PSs that have been recently defined in the Directive 2013/39/EU is shown in *Figure 6.* It is notorious that a quite significant number of studies for some compounds has been performed (e.g., benzene, followed by nonylphenol and chlorpyrifos), whereas there is still a considerable number of them without information on removal by CWs (e.g., some pesticides, such as aldrin, isodrin and endrin, among other PSs).

Concerning the group of substances *(Figure 6),* pesticides are the most studied PSs in CWs, including triazine (25.2%), organophosphorus (9.8%), organochlorine (9.0%), phenylurea (7.3%) and other classes of pesticides (1.6%). There are also several works studying the removal of solvents by CWs (24.4%), and among them benzene is the most representative compound (n = 25), followed by 1,2-

dichloroethane (n = 2), dichloromethane (n = 1), trichloromethane (n = 1) and pentachlorobenzene (n = 1).

Industrial compounds represent 17.9% of the works conducted by using CWs, in the following decreasing order of number of studies: nonylphenol > DEHP > octylphenol ≈ PFOS. PAHs, dioxins and dioxin-like compounds as well as organometallic compounds, are the PSs least reported, each representing 1.6% of the published works.

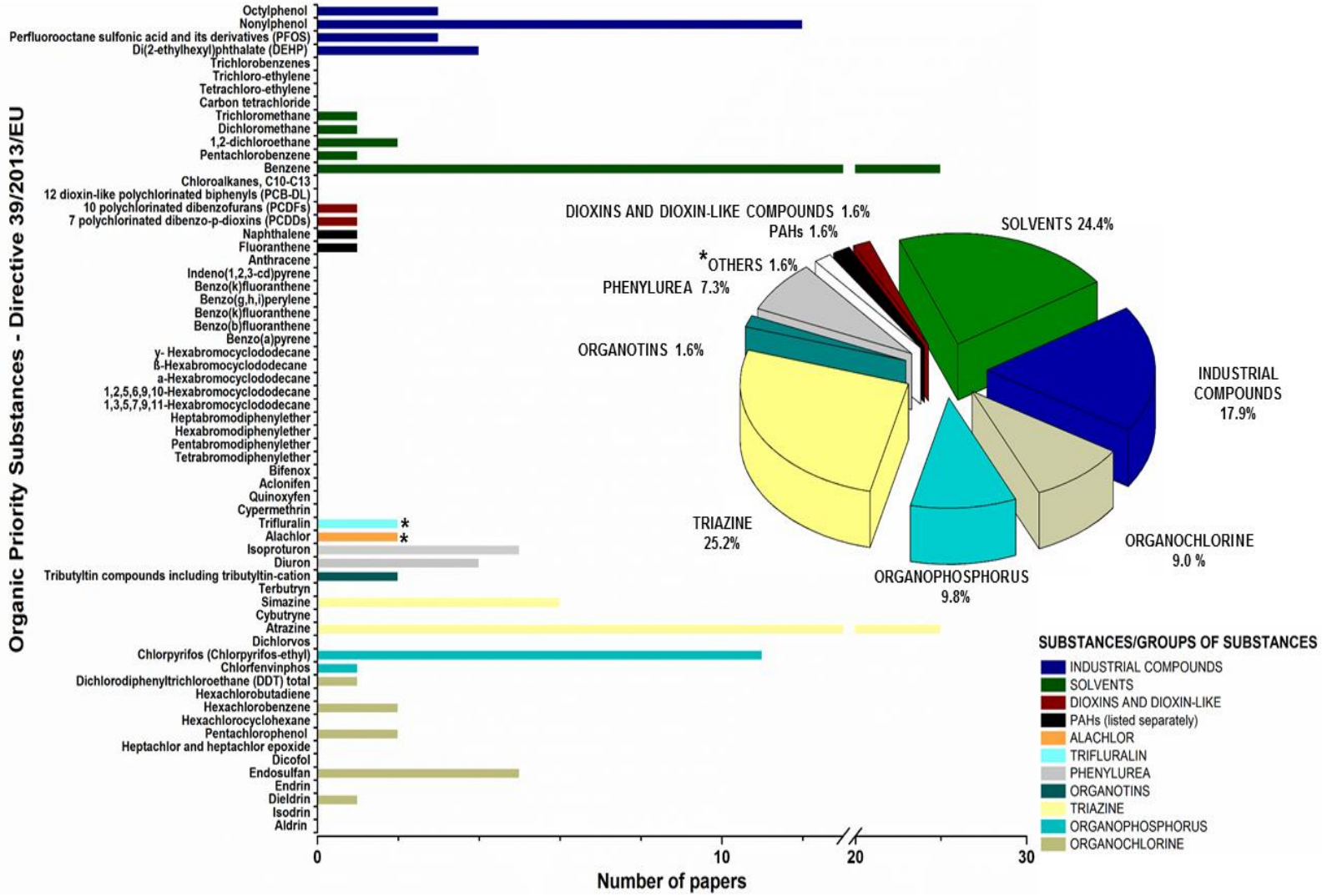


Figure 6. Number and percentage of publications dealing with removal of organic PSs listed in Directive 2013/39/EU by CWs, based on the *Scopus* database search by using as keywords the name of each PS and constructed wetland (accessed until June 2016).

The inlet concentrations of PSs in CWs are shown in *Figure 7 and 8*, respectively when dealing with non-spiked or spiked samples. For non-spiked samples, it can be observed that the removal of benzene, nonylphenol and chlorpyrifos was studied at the highest initial concentrations, and at different levels of magnitude for each organic PS, respectively, up to tens of mg L^{-1}, at few mg L^{-1} and at µg L^{-1} levels. The pesticides isoproturon and diuron were studied up to 88 and 45 and µg L^{-1}, respectively, whereas concentrations of few µg L^{-1} were found in the non-spiked inlet samples for atrazine, simazine, pentachlorophenol and endosulfan, as well as for the industrial compounds octylphenol, PFOS and DEHP, and for solvent trichloromethane.

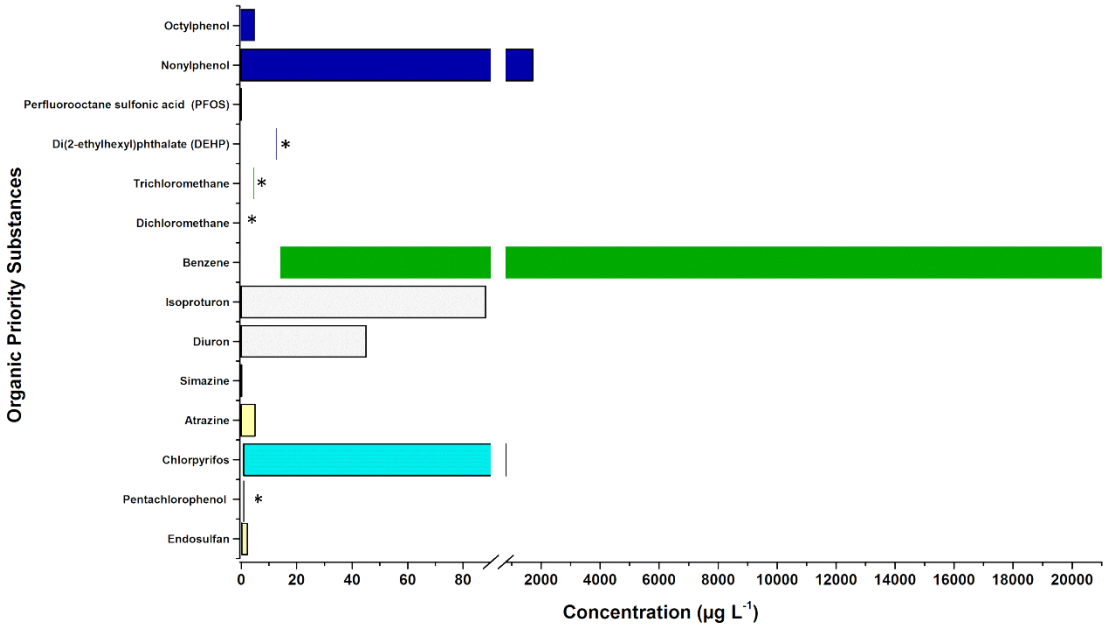


Figure 7. Concentration range (µg L^{-1}) of organic PSs in non-spiked samples used as inlet solution in the CWs experiments (* only one initial concentration was found).

Works on CWs to remove such type of compounds were also performed by spiking the inlet solution with different concentrations of the pollutants. Considering the decreasing order of concentrations found for PSs in the non-spiked effluents *(Figure 7)* used as inlet solutions in the CWs (benzene > nonylphenol > chlorpyrifos > isoproturon > diuron > DEHP > octylphenol > atrazine > trichloromethane >endosulfan > pentachlorophenol > simazine > dichloromethane > PFOS), it can be concluded that the spiked concentrations already studied *(Figure 8)* (benzene > simazine > atrazine > PFOS > alachlor = diuron = pentachlorophenol > endosulfan > nonylphenol >>> chlorpyrifos > hexachlorobenzene) do not really match the non-spiked samples. For instance, nonylphenol, one of the PSs which was found at the highest range of concentrations in the non-spiked effluents used for evaluation of its removal by CWs, was the compound studied at the lowest concentration for spiked experiments. Chlorpyrifos and isoproturon, also found at high concentrations in non-spiked treatments employing CWs, were not considered in any spiked experiment. Simazine, which is normally found in realistic samples at lower concentrations than the other triazine pesticide atrazine, has been studied at higher concentrations than atrazine in works using spiked inlet solutions. In addition, benzene and the triazine pesticides (atrazine and simazine) were those spiked at the highest concentrations, at 1 g L^{-1} and up to hundreds of mg L^{-1}, respectively *(Figure 8)*. As shown in *Figure 8*, the removal of atrazine by CWs has been evaluated at the largest range of

concentrations. The elimination by CWs of the pesticides alachlor and diuron was assessed only in one study for each compound, by spiking the inlet solution with one of these compounds. Only one study using spiked water is available for each of the following compounds: nonylphenol, PFOS (industrial compounds) and pentachlorobenzene (solvent). The pesticide chlorpyrifos was studied up to 3 µg L^{-1} and hexachlorobenxene up to 2.5 µg L^{-1}.

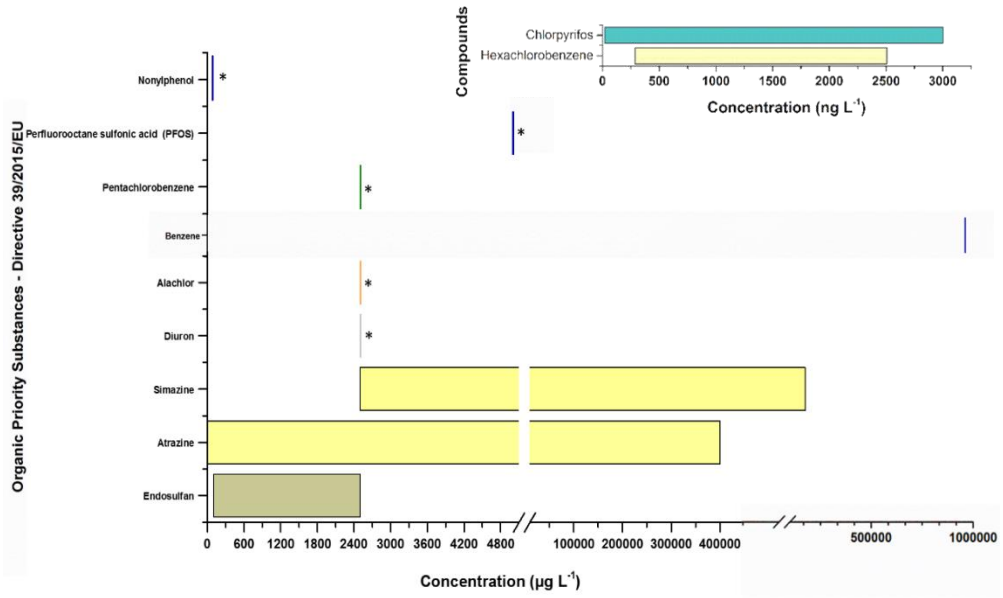


Figure 8. Concentration range (µg L^{-1}; ng L^{-1}) of organic PSs in spiked samples used as inlet solution in the CWs experiments (* only one spiked concentration was found).

The performance of CWs to eliminate these MPs is shown as removal efficiencies in *Figure 9*. The industrial compounds octylphenol, nonylphenol and PFOS were removed at poor to high extent, whereas DEHP was eliminated between 19 and 49% *(Figure 9)*. The pesticides alachlor (80%), pentachlorophenol (89-94%) and endosulfan (> 80%) were highly removed by CWs and the concentration of isoproturon and hexachlorobenzene was moderately reduced by 45% and 67-75%, respectively. The performance observed for the elimination of diuron varied up to 55%. The triazine pesticides had a different behaviour, with simazine being removed between 20 and 60% and atrazine with a very dissimilar elimination, with either inefficient or complete removal. The decrease on the concentration of chlorpyrifos in the studied CWs described in the literature was similar to that observed for atrazine, from almost none to a total elimination. In any case, a comparison between different studies and the respective removal achieved for different compounds is merely indicative since different CWs systems and operating conditions were tested in most of these publications.

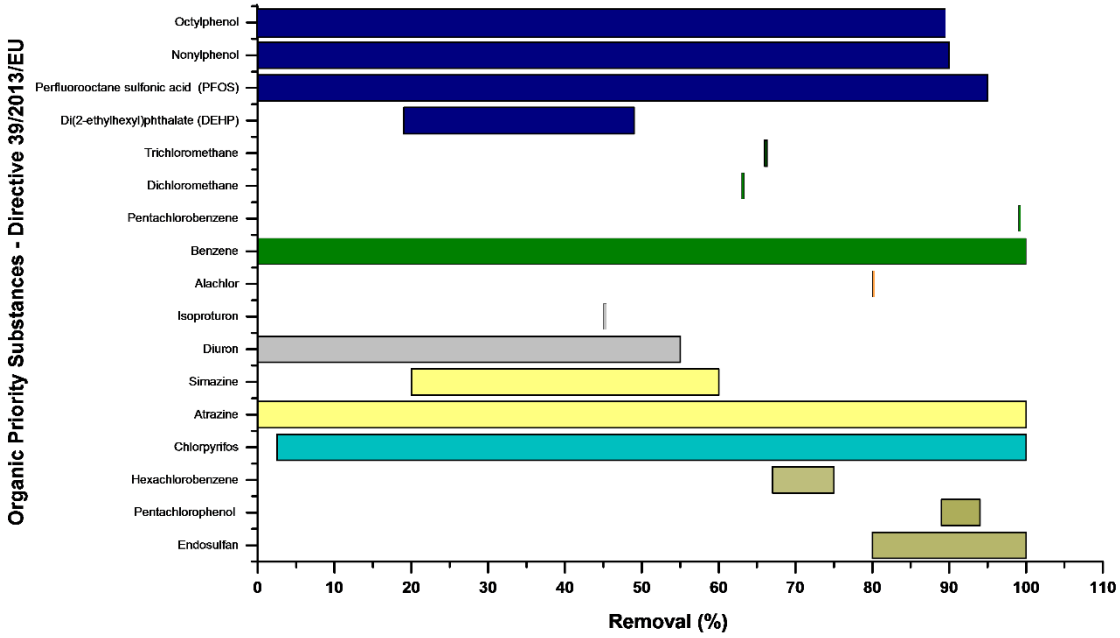


Figure 9. Removals range (%) verified for sixteen organic PSs.

2.2. Studies dealing with the removal of Watch List compounds (CECs) by CWs

The studies on the removal of CECs (Watch List of EU Decision 2015/495/EU) by CWs are represented by substances/groups of substances in *Figure 10*. The most studied substances were diclofenac (43.8%), followed by macrolide antibiotics (erythromycin, clarithromycin and azithromycin), E2 and E1 (each representing 15.6% of the total studies), and the synthetic hormone EE2 (7.8%). It is interesting to note that diclofenac, E2 and EE2, earlier (in 2013) suggested in Directive 39/2013/EU as compounds to be included in the first Watch List, represent together almost 70% of the published works on CWs, in contrast with the others only identified in 2015, demonstrating the relevance of these compounds in the environment. The three macrolide antibiotics erythromycin, clarithromycin and azithromycin are still poorly studied (15.6% for the three drugs), even if they have a potential negative impact on the environment. Neonicotinoids (imidacloprid) are only reported in one study. The other CECs included in the Watch List were not considered in any study, namely the pesticides thiacloprid, thiamethoxam, clothianidin, acetamiprid, methiocarb, oxadiazon and triallate, the UV filter 2-ethylhexyl 4-methoxycinnamate and the antioxidant 2,6-di-tert-butyl-4-methylphenol commonly used as food additive.

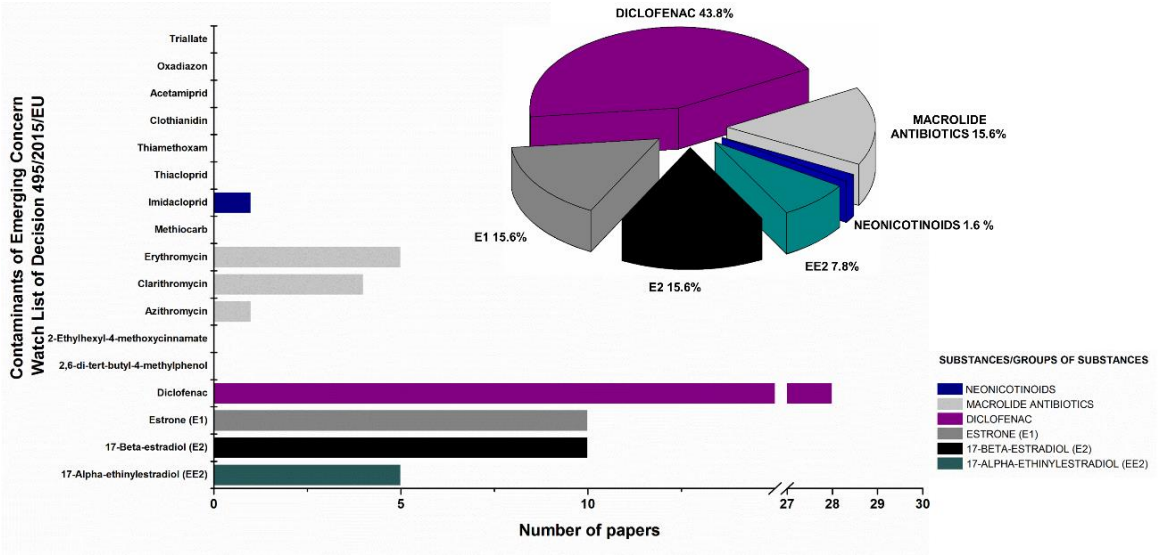


Figure 10. Number and percentage of publications dealing with removal of CECs listed in Watch List of Decision 495/2015/EU by CWs, based on the *Scopus* database search by using as keywords the name of each CEC and constructed wetland (accessed until June 2016).

The concentrations of the CECs included in the Watch List are summarized in *Figure 11 and 12*, respectively for non-spiked and spiked water samples used as inlet solution in CWs. In both cases, diclofenac was the compound found with the highest range of concentrations. Its removal was evaluated in spiked inlet solutions using concentrations of the same order of magnitude than those found in realistic samples, i.e., up to 37 μg L^{-1} *(Figure 11)*, but higher concentrations were also tested in spiked experiments, i.e., up to 5000 μg L^{-1} *(Figure 12)*. The macrolide antibiotics erythromycin and clarithromycin were found in the effluents and their elimination was assessed at concentrations ≤ than 0.3 μg L^{-1}; however, the spiked experiments were performed using higher concentrations (between 0.4 μg L^{-1} and 2 μg L^{-1}) and also including azithromycin (ca. 0.3 μg L^{-1}). The three estrogens were studied in non-spiked effluents at concentrations ≤ than 0.2 μg L^{-1}, but only E2 was reported in spiked experiments, using concentrations quite higher (2250 μg L^{-1}). Besides the compounds found in the non-spiked samples treated by CWs, only the neonicotinoid imidacloprid was also studied using spiked inlet solutions.

Overall, CECs listed in the Watch List were found at lower concentrations in the realistic samples when compared with PSs (Section 2.2.1). The spiked experiments were also designed using inlet solutions with lower concentrations of CECs than those of PSs.

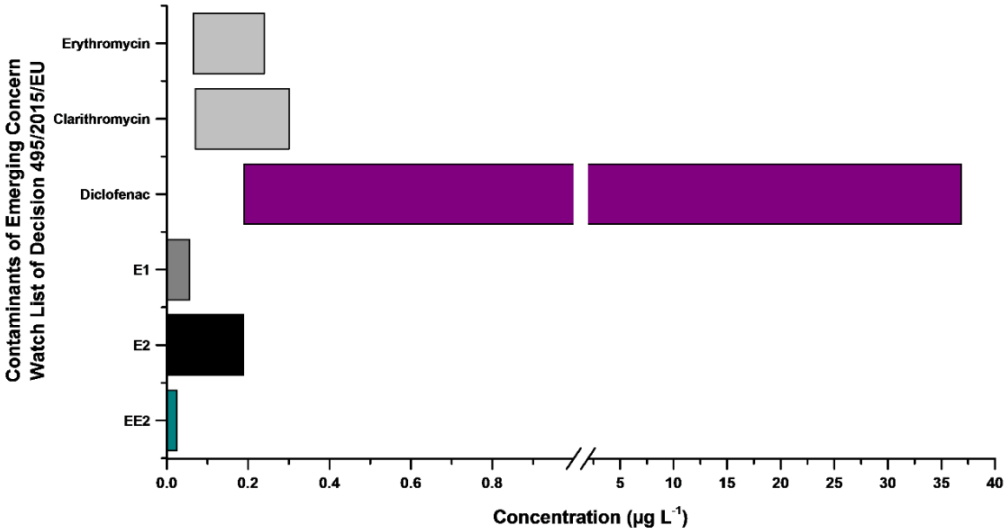


Figure 11. Concentration range (µg L^{-1}) of CECs in non-spiked samples used as inlet solution in the CWs experiments.

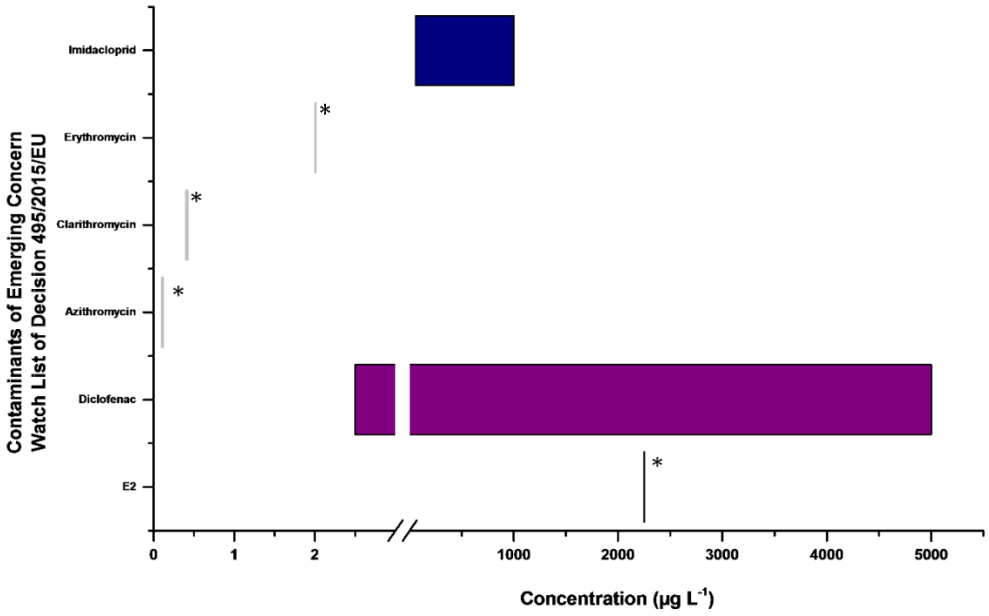


Figure 12. Concentration range (µg L^{-1}) of CECs in spiked samples used as inlet solution in the CWs experiments (* only one spiked concentration was found).

Regarding the performance of CWs to remove CECs, *Figure 13* suggests that diclofenac and the antibiotics erythromycin and clarithromycin are more difficult to remove than the other compounds at the operating conditions already employed in the literature. The three estrogens were eliminated up to 100%, with a minimal removal of 14% for E1, 36% for E2 and 17% for EE2. The spiked concentration of the neonicotinoid pesticide imidacloprid was reduced by ≥ 70%.

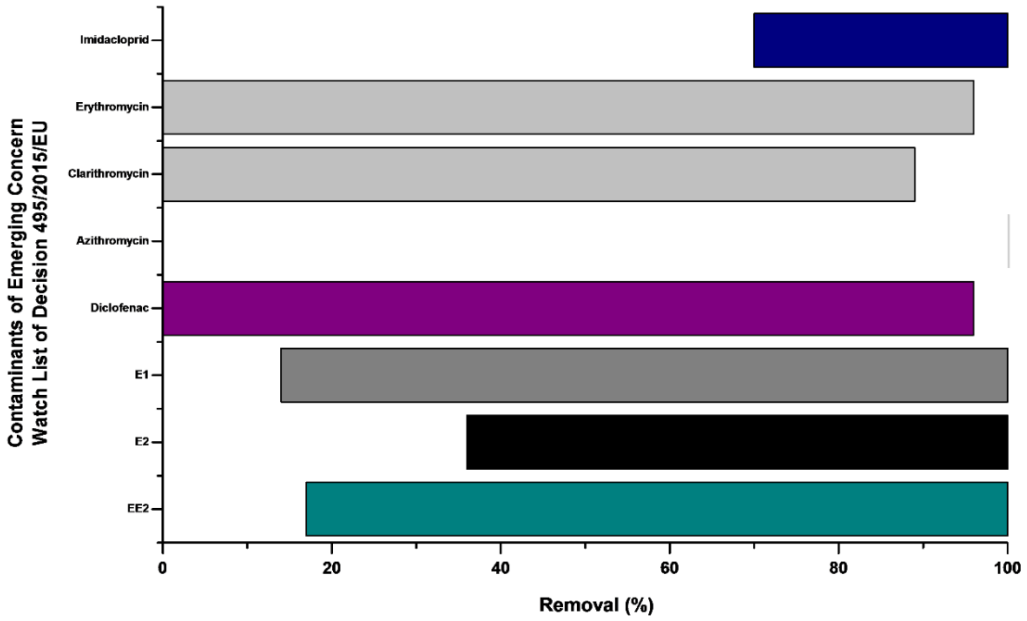


Figure 13. Removal range (%) verified for eight CECs.

By the analysis of *Figure 9 and 13* it is possible to verify that in most cases the already studied PSs and CECs can be highly removed by CWs, exceptions being found for DEHP, isoproturon, diuron and simazine, with removals always lower than 60%. As referred in Section 1.5.1., several factors can affect the removal mechanisms on the CWs, and hence distinct conditions tested can have as consequence variable efficiencies. As the performance of CWs to remove MPs is a recent research field, multiple parameters affecting the CW systems and treatment efficiencies, should be evaluated. The characteristics of the effluents may also influence the treatment occurring in the CWs, which also explains the different results for the same contaminant.

3
Experimental

3.1. Chemicals and materials

A set of 21 organic compounds was selected as target MPs to be determined in aquaculture influents and effluents. Some of them are included in the Directive 2013/39/EU as PSs (perfluorooctanesulfonic acid (PFOS), atrazine, simazine, isoproturon) and in the Watch List of Commission Decision 2015/495/EU (diclofenac, erythromycin, clarithromycin, azithromycin, methiocarb, imidacloprid, thiacloprid, thiametoxam, clothianidin, acetamiprid, 2-ethylhexyl 4-methoxycinnamate). Other group of compounds was selected based on their recalcitrance in surface water (carbamazepine, clofibric acid) and typical administration as veterinary drugs for aquaculture practices (ofloxacin, enrofloxacin, ceftiofur, ivermectin). Table 1 shows the class, structure, molecular weight (Mw) and p*K*a of the target analytes. All reference standards (98% purity) were acquired from Sigma-Aldrich (Steinhein, Germany). Surrogate standards (atrazine–d5, methiocarb-d3, clothianidin-d3, acetamiprid-d3, diclofenac-d4, azithromycin-d3 and ofloxacin-d3) were purchased from Sigma-Aldrich (Steinhein, Germany).

Each reference standard was dissolved in methanol to obtain stock solutions with a concentration of approximately 1000 mg L^{-1}. These solutions were then diluted in the same solvent to prepare individual standard solutions for each compound (10 mg L^{-1}) and two working standard solutions containing all the analytes at 10 mg L^{-1} and 0.3 mg L^{-1}, used for MS/MS and SPE-UHPLC optimization, respectively. A working solution containing 10 mg L^{-1} of each isotopically labeled internal standard was prepared by dilution in ethanol.

Ethanol was purchased from Fisher Scientific (Leicestershire, UK). Methanol and acetonitrile were acquired from VWR International (Fontenay-sous-Bois, France). Ammonium acetate, ammonium hydroxide 25%, sulphuric acid and formic acid were purchased from Merck (Darmstadt, Germany). A Milli-Q water system was used to provide ultrapure water. MS-grade solvents were filtrated with 0.22 µm nylon membrane filters (Membrane Solutions, Texas, USA). Cartridges tested for SPE were Oasis® HLB (Hydrophilic-Lipophilic-Balanced), Oasis® MCX (Mixed-mode Cation eXchange) and Oasis® MAX (Mixed-mode Anion-eXchange) (150 mg, 6 mL), purchased from Waters (Milford, Massachusetts, USA). A pH meter pHenomenal® pH 1100L (VWR, Germany) was used for pH adjustments.

Table 1. Group of compounds studied by class and inclusion criteria: analyte, structure, molecular weight (Mw) and p*K*a

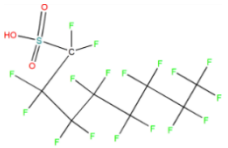
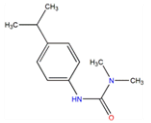
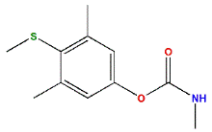
Class and sub class	Inclusion Criteria				Analyte	Structure	Mw (g mol^{-1})	p*K*a
	PS	CEC	Vet. Drugs	Recalcitrance				
Industrial compound	×				Perfluorooctanesulfonic acid *(PFOS)*		500.13	0.14
Pesticides								
Triazine	×				Atrazine		215.68	4.14
	×				Simazine		201.66	1.62
Phenylurea	×				Isoproturon		206.28	n.a.
Insecticide		×			Metiocarb		225.31	12.2

Table 1. Continued.

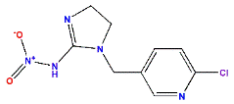
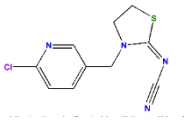
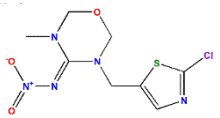
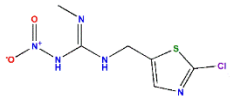
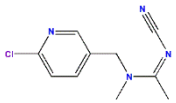
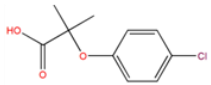
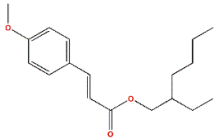
Class and sub class	Inclusion Criteria				Analyte	Structure	Mw (g mol^{-1})	p*Ka*
	PS	CEC	Vet. Drugs	Recalcitrance				
Pesticides								
		×			Imidacloprid		255.66	11.1
		×			Thiacloprid		252.72	n.a.
Neonicotinoids		×			Thiamethoxam		291.71	n.a.
		×			Clothianidin		249.68	11.09
		×			Acetamiprid		222.67	0.70
Herbicide				×	Clofibric Acid		214.65	3.00
Organic UV Filter		×			2-Ethylhexyl 4-methoxycinnamate *(EHMC)*		290.40	n.a.

Table 1. Continued.

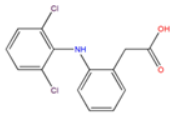
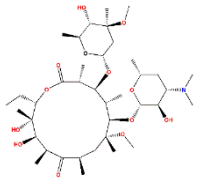
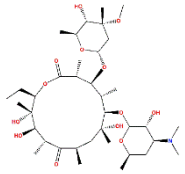
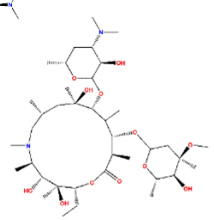
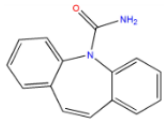
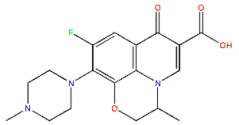
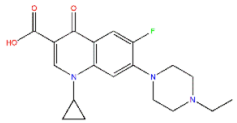
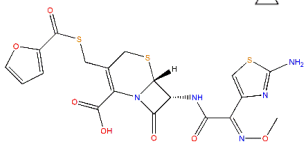
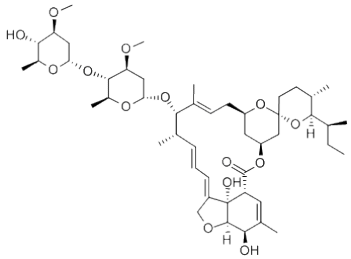
Class and sub class	Inclusion Criteria				Analyte	Structure	Mw (g mol^{-1})	p*K*a
	PS	CEC	Vet. Drugs	Recalcitrance				
Pharmaceuticals								
Anti-inflammatory		×			Diclofenac		296.14	4.15
		×			Erythromycin		733.93	8.90
Macrolide Antibiotics		×			Clarithromycin		747.95	8.99
		×			Azithromycin		748.98	8.74
Psychiatric drug				×	Carbamazepine		236.27	13.94

Table 1. Continued.

Class and sub class	Inclusion Criteria				Analyte	Structure	Mw (g mol^{-1})	p*Ka*
	PS	CEC	Vet. Drugs	Recalcitrance				
Pharmaceuticals								
Antibiotics			×		Ofloxacin		361.37	5.23
			×		Enrofloxacin		359.39	6.43
			×		Ceftiofur		523.56	2.5
Anthelmintic			×		Ivermectin		875.09	12.47

3.2. Analytical method for determination of organic MPs

3.2.1. Solid phase extraction (SPE)

SPE was optimized for some of the target contaminants under analysis, namely PSs included in Directive 39/2015/EU (PFOS, atrazine, simazine, isoproturon), the recalcitrant compounds (clofibric acid and carbamazepine) and the veterinary drugs (ofloxacin, enrofloxacin, ceftiofur and ivermectin). Regarding the SPE conditions for the Watch List compounds (diclofenac, 2-ethylhexyl 4-methoxycinnamate (EHMC), erythromycin, clarithromycin, azithromycin, methiocarb, imidacloprid, thiacloprid, thiamethoxam, clothianidin and acetamiprid), they were previously optimized in our laboratory [164]. Therefore, SPE was optimized for a total of 10 compounds in the present work, while previously optimized SPE conditions were used to determine a total of 11 compounds from the Watch List, being possible to determine a total of 21 MPs. Surface water collected from the source of Sousa River located in Portugal, was used as matrix for SPE optimization, which was performed using an extraction manifold of 20 positions provided by Waters (Milford, MA, USA) *(Figure 14).*

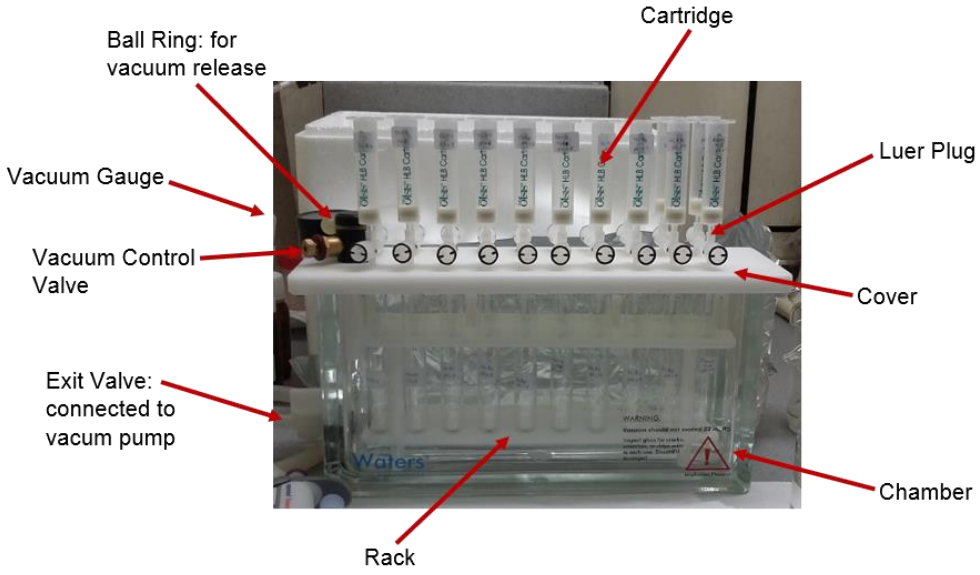


Figure 14. Waters 20-Position Extraction Manifold (Adapted from ref.[165]).

In order to maximize the extraction recoveries, different cartridges (Oasis® HLB, MCX and MAX), solvents (methanol, ethanol and acetonitrile), of sample pH (3, 7, 9) and sample volumes (250, 500, 1000 mL) were tested. HLB cartridges were conditioned sequentially with 4 mL of methanol, ethanol or acetonitrile and 4 mL of ultrapure water at flow rate of 1 mL min^{-1}. MCX and MAX cartridges were conditioned equally, but only methanol was tested as organic solvent. A standard solution of 0.3 mg L^{-1} was used to spike triplicates of 250 mL water samples and blank samples were also prepared. The sample pH was adjusted with ammonium hydroxide and sulphuric acid, before loading in HLB (pH 3, 7, 9), MCX (pH 3) or MAX (pH 9). Sorbent washing was performed with 4 mL of ultrapure water (HLB), 2 % formic acid aqueous solution (MCX) and 5 % ammonium hydroxide aqueous solution (MAX), followed by 45 min of vacuum drying. Elution step of HLB cartridges was performed with 4 mL methanol, ethanol or acetonitrile, whereas MCX and MAX cartridges were eluted twice: 4 mL of methanol to extract the neutral and weak acidic compounds and 5 % ammonium hydroxide methanolic solution to elute the basic compounds (MCX); 4 mL of methanol to extract the neutral and weak basic compounds and 4 mL of 2 % formic acid methanolic solution to elute the acid compounds (MAX). The

resulting extracts were evaporated to dryness in a CentriVap® Concentrator, purchased from LABCONCO (Kansas City, USA). Reconstitution of the dry residues was performed in 250 µL of methanol (ethanol or acetonitrile for HLB) and filtered by 0.22 µm polytetrafluoroethylene (PTFE) syringe filters (Membrane Solutions, Texas, USA). As referred, the percolating volume of sample was also optimized, after selecting the cartridge (HLB), solvent (ethanol) and pH (3).

The peak areas of the compounds extracted from the spiked samples were compared with the ethanolic solutions containing the target analytes at the same theoretical concentrations of the extracts of spiked matrix, to determine the recovery of each compound in SPE procedure. Blanks samples were also extracted and analysed to subtract the detected target compounds from those obtained with spiked matrix.

3.2.2. Ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) analysis

A Shimadzu Corporation apparatus, coupling LC and MS detection, was used for sample analysis *(Figure 15)*. It consists on an UHPLC equipament (Nexera), including two pumps (LC-30AD), an autosampler (SIL-30AC), an oven (CTO-20AC), a degasser (DGU-20A 5R) and a system controller (CBM-20A) with proper software (LC Solution Verson 5.41SP1). A triple quadrupole mass spectrometer detector (Ultra Fast Mass Spectometry series LCMS-8040) is coupled to the UHPLC. The ionization source used was the ESI, operating in positive and negative modes, and the collision induced dissociation gas (CID) was argon at 230 kPa.The chromatographic column was a Kinetex™ 1.7 µm XB-C18 100 Å (100 × 2.1 mm, i.d.) provided by Phenomenex, Inc. (California, USA).

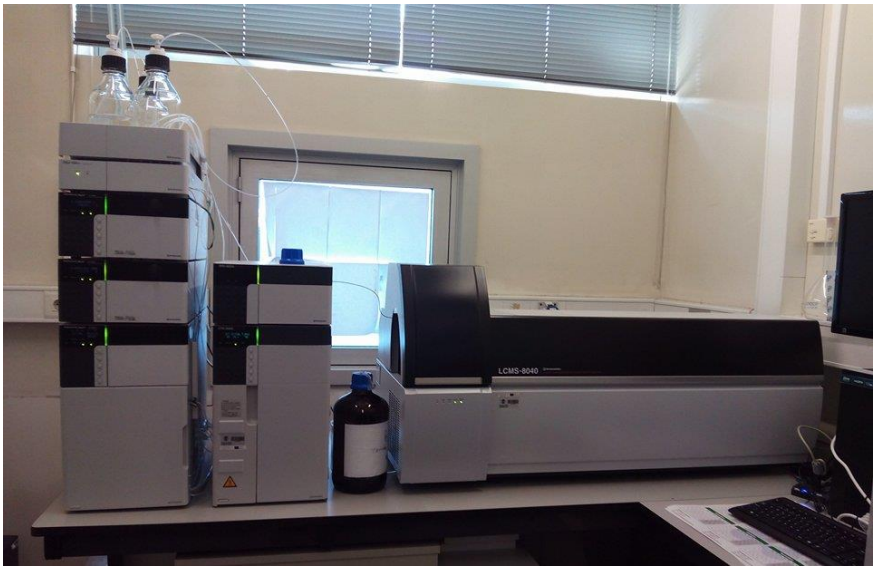


Figure 15. Equipment used for LC-MS/MS analysis.

MS optimization was performed in the present work for veterinary drugs (Method A), whereas already optimized MS conditions [164, 166] were used for all other classes of target compounds (Method B).

In Method A, the choice of the precursor ion, the most abundant fragments and MS parameters (declustering potential, collision energy and collision cell exit potential) were performed through direct

injection of 10 µL of the individual standard solutions (10 mg L^{-1}). The most abundant fragment (SRM1) ion was used as quantifier of MPs and the second most abundant (SRM2) as qualifier. Other parameters such as capillary voltage (0.5, 1.5, 2.5, 3.5 and 4.5 kV), drying gas flow (10.0, 12.5 and 15.0 dm^3 min^{-1}), nebulizing gas flow (1.0, 1.5, 2.0, 2.5 and 3.0 dm^3 min^{-1}), desolvation temperature (200, 225, 250, 275 and 300 º C) and source temperature (250, 300, 350, 400 and 450 º C) were also optimized injecting a working standard solution of 10 mg L^{-1}. Different mobile phases were tested and a gradient of methanol and water containing 0.1% formic acid (initial conditions: 60/40, v/v) was chosen, performed at a flow rate of 0.20 mL min^{-1}. The gradient was programmed as follows: 60% for 3 min; a linear gradient from 60% to 95% in 1 min (held for 5 min), a linear gradient from 95% to 60% in 0.5 min, and finally an equilibration time of 3.5 min, completing a total run time of 13 min. For overall analyses, the temperatures of column oven and autosampler were set at 35 ºC and 4 ºC, respectively.

Regarding the PSs (PFOS, atrazine, simazine, isoproturon) and the recalcitrant compounds (clofibric acid and carbamazepine), i.e., Method B, the conditions were optimized in another work [164] and included in a previously developed method to analyze the 11 Watch List compounds [166], using a mobile phase of methanol/water (75, 25, v/v) performed at gradient mode with a flow rate of 0.25 mL min^{-1}. The values of capillary voltage, drying gas flow, nebulizing gas flow, desolvation temperature and source temperature were, respectively: 4.5 kV, 12.5 dm^3 min^{-1}, 3.0 dm^3 min^{-1}, 250 ºC, 400 ºC.

3.2.3. Validation parameters

The developed analytical method was validated according to the international guidelines [167] and previous works [168-170], considering the parameters of selectivity, linearity and range, limits of detection and quantification, accuracy, recovery and precision. The validation process was carried out using surface water collected at the same point of that used for SPE optimization, i.e., source of Sousa River located in Portugal.

Depending on the sensibility of the analytes, two levels of concentrations were established for validation. A standard solution containing all the target analytes at 200 µg L^{-1} except for ofloxacin, enrofloxacin, ceftiofur and ivermectin, which were at 800 µg L^{1}, was prepared by diluting stock solutions in ethanol. The matrix (500 mL) was spiked with the same standard solution and the pH was adjusted to 3. Prior to extraction, 20 µL of a working internal standard solution (10 mg L^{-1}) was added to each sample. Selectivity was verified by comparing the chromatogram of an ethanolic solution containing all standards, standards extracted from the spiked effluents and non-spiked effluents (blank extracts). After SPE, the reconstituted extracts were analyzed and internal calibration curves were performed for each compound to assess linearity and range. Method detection (MDL) and quantification (MQL) limits were determined through evaluation of the signal of three blanks extracts, three extracts of spiked matrices and three ethanolic solutions for which the signal of the target compounds, the noise (before and after their retention time) and the signal of internal standards, were registered. The standard deviation of the signal was divided by the slope of calibration curves and multiplied by 3.3 or 10, to calculate MDL or MQL, respectively. Instrument detection (IDL) and quantification (IQL) limits resulted from the multiplication by the pre-concentration factor (2000). Three quality control (QC) standard solutions, each prepared in triplicate, were used for the recovery assays, which consisted in extracts of matrix spiked at three levels of concentration for each compound (4.5, 45 and 90 or 18,180 and 360 µg L^{-1}). The peak areas of the compounds extracted from the spiked samples were compared with the ethanolic solutions containing the target analytes at the same theoretical concentrations of the extracts of spiked matrix, to determine the recovery of each compound in SPE procedure. Recovery was calculated as referred in Section 3.2.1.

The QC solutions above mentioned were also applied to evaluate the method accuracy and precision (intra- and inter-batch). In order to determine the accuracy, the concentrations of the analytes in the SPE extracts (calculated using the calibration curves) were divided by the nominal concentration. Intra-day and inter-day precision was assessed by the evaluation of the relative standard deviation for each QC level.

3.2.4. Matrix Effect

In order to determine the matrix effect (ME), the post-extraction analytical method was performed [170, 171]. Analysis of three spiked blanks extracts (A) with a concentration of 45 or 180 µg L^{-1}, depending on the compounds, and analysis of three blanks extracts (B), were carried out. For each compound, the ME was determined as the ratio of the peak areas after subtracting the blank signal (A – B), and the peak areas of three standard ethanolic solutions with the same teorethical concentration of the spiked blanks extracts (E). The ME can be expressed by the equation: ME (%) = (A – B)/E × 100 [170, 171]. Values higher than 100% indicate ionization enhancement and values lower than 100% show ionization suppression; a ME of 100% indicates the absence of matrix effect.

3.3. Sampling of water and plants

3.3.1. Aquaculture influents and effluents

To assess the removal of the MPs by CW and ozonation processes, water samples were collected in a freshwater fish farm located in Portugal. The aquaculture farm occurs in artificial channels (raceways), built very close to a river, where only one species of fish (trout) is produced. The sampling was performed in March and May 2016, including the harvesting of inlet and outlet (effluent) water to determine the MPs present in these water samples and to study the treatment of the effluents by using CWs and ozonation processes *(Figure 16)*. The samples were transported to the laboratory and stored at 4 ºC until extraction (influent and effluent).

Figure 16. Sampling at inlet (left) and outlet of aquaculture (right).

3.3.2. Plants and support matrix

For assembling the CWs systems, indigeneous plants (*Phragmites australis*) and the sediments involving their roots were collected in the riverbank of a River of Portugal in May 2016 *(Figure 17).*

Figure 17. Collection site of plants and sediments for the CWs creation.

On sampling site, the solid fraction was separated from the plants roots, by washing with river water. Plants and sediments were transported to the laboratory, where they were used for application on CWs systems.

3.4. Treatments processes

3.4.1. Constructed wetlands experiments

CWs systems were set up at bench-scale to evaluate their efficiency on the MPs removal. In the specific case of this work, the CWs were partially built already, as result of a PhD thesis developed at CIIMAR, thus the collected plants and their support matrix were assembled, using plastic boxes (0.4 m x 0.3 m x 0.3 m) previously filled with a first layer of gravel (4 cm depth) and a second layer of lava rock (2 cm depth), according to described elsewhere [172]. Plants and their support matrix were placed over the substrate described and rigorously distributed to originate three similar CWs replicates. The boxes were covered with aluminium foil to simulate a real system, preventing the light irradiation and consequently the photodegradation of compounds. To enable the collection of treated effluent, a plastic tap was coupled at the bottom of the boxes. The *Figure 18 and 19* demonstrate the CWs assembling [172].

Figure 18. CWs assembling steps pictures (Adapted from ref.[172])

Figure 19. Plants and sediment introduced in the prepared boxes.

In the first three days, the triplicate CWs were irrigated with a nutrient solution *(*Appendix A3*)* to maintain good nutritional conditions for plants and to guarantee the same conditions of their support matrices. Two sets of experiments were performed using CWs. The first set was carried out by supplementing 3 CWs with non-spiked aquaculture effluents (1.5 L). The second set of experiments was performed using aquaculture effluents (1.5 L) spiked with 15 µL of a 10 mg L^{-1} solution containing 10 MPs (atrazine, simazine, clofibric acid, erythromycin, carbamazepine, ofloxacin, enrofloxacin, ceftiofur, ivermectin and EHMC), resulting in a 100 ng L^{-1} of each compound in the effluent. Each set of experiments occurred during one week, which started with the addition of the water resulting from the aquaculture activity (1.5 L) to the CWs, i.e. as the inlet of the CWs systems. Every day, the effluent of each CW was collected through the tap and re-introduced in the same system, in order to prevent the development of anoxic areas and to favor the bacterial degradation (batch mode). Deionized water was added whenever necessary to compensate the water loss by evaporation. At the end of each set of experiments, the CW effluent was collected and deionized water was added up to 1.5 L, equaling the initial volume. The effluent was then treated by the ozonation process in order to study the possible removal of the MPs detected after CW treatment. The *Figure 20 and 21* illustrate some steps performed in the CWs experiments.

Figure 20. Addition of nutrient solution (left) and collection of treat effluent (right).

Figure 21. Addition of deionized water to complete 1.5L in the final samples (left). Treat samples by 3 CWs and samples for ions quantification.

3.4.2. Ozonation

Ozonation experiments were carried out at bench-scale *(Figure 22)* to evaluate the removal of organic MPs that were not fully eliminated by CWs systems. Each effluent sample of the 3 CWs of both sets of experiments (spiked and non-spiked samples) were treated by ozonation. These assays were performed during 10 min in a 1 L reactor loaded with 600 mL of effluent samples collected in the CWs and with a constant magnetic stirring of 400 rpm.

Figure 22. Ozonation experiments at bench-sacle.

Ozone was produced using a BMT 802X ozone generator (which generates ozone from pure oxygen) and its inlet concentration was monitored with a BMT 964 ozone analyser. It was established a constant ozone flow rate of 90 cm^3 min^{-1} and a constant inlet concentration of 50 g m^{-3}[164]. The ozone leaving the reactor in the gas phase was also monitored. In order to remove the dissolved oxygen, at the end of each ozonation experiment, the gas stream was replaced by oxygen for 30 min, at the same flow rate (150 cm^3 min^{-1}).

3.5. Characterization of water samples

The water samples were analysed before and after aquaculture as well as before and after CW and ozonation processes. 21 MPs were analyzed by SPE-UHPLC-MS/MS, as described in Section 3.2. The dissolved organic carbon (DOC) was determined in a Shimadzu apparatus by the difference of the measured total carbon (TC) and inorganic carbon (IC) in the filtered samples. The concentration of ammonium, nitrate, nitrite, sulphate, chloride, phosphate, bromide, bromate, potassium, sodium, calcium and magnesium, was determined by ionic chromatography using a Metrohm 881 Crompaed IC Pro equipment A Metrostep C4 Cationic Change Column (250 mm × 4.01 mm) was employed for quantification of cations and a Metrostep A Supp 7 Anionic Change Column (250 mm × 4.01 mm) for anions.

4
Results and Discussion

4.1. Analytical method

4.1.1. SPE optimization

In the present work, a detailed SPE optimization study was carried out to maximise the extraction of 10 MPs (Watch List compounds were already optimized as described in Section 3.2.1), in order to obtain a high recovery from water samples. In this sense, different parameters were evaluated, namely the sample pH, the extraction solvent, the type of cartridge and the sample volume. The recoveries obtained for each target analyte, by varying these parameters, are described below.

4.1.1.1. Sample pH

The influence of sample pH on the recovery of the analytes was studied (pH 3, 7 and 9). For this purpose, Oasis® HLB (Hydrophilic-Lipophilic-Balanced) cartridges were used to extract 250 mL of spiked water samples. Methanol was used as cartridge conditioning and eluting solvent. The assays were performed in triplicate and the obtained results are shown in *Figure 23*.

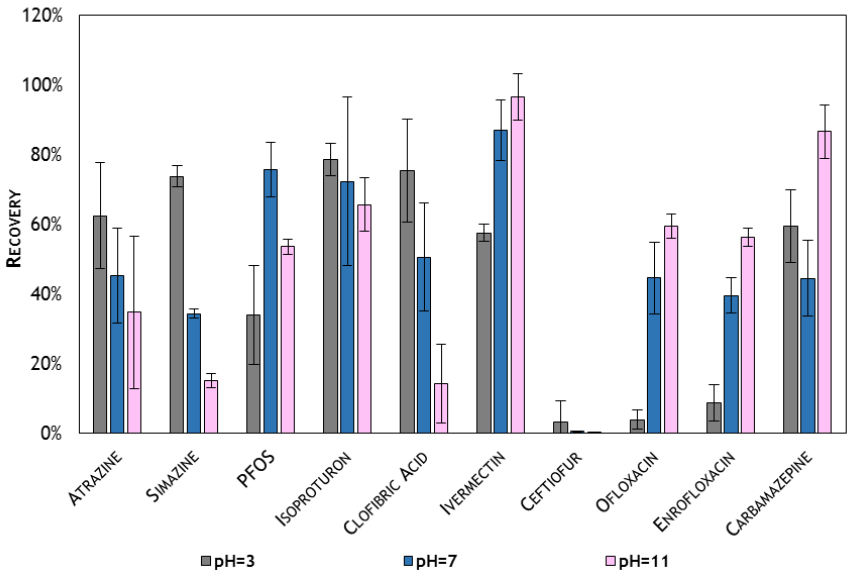


Figure 23. Recoveries obtained for 10 MPs using different sample pH (3, 7 and 9) to extract 250 mL of spiked water samples through Oasis® HLB cartridges and using methanol as solvent.

It is possible to verify that the recovery of the compounds varied with the pH value, with atrazine, simazine, isoproturon, clofibric acid and ceftiofur being better recovered from acidified samples, while others are better recovered using basic (ivermectin, ofloxacin, enrofloxacin and carbamazepine) or neutral pH (PFOS). In order to analyze the samples using a single SPE procedure, the sample pH 3 was selected since it provides higher recoveries for most compounds, favoring their extraction.

4.1.1.2. Eluting solvent

The conditioning and eluting solvents were tested after pH selection. Oasis® HLB cartridges were employed to extract the same water volume at pH 3, using ethanol or acetonitrile as eluting solvents instead of methanol. The results are shown in *Figure 24*.

Ethanol was considered the most adequate solvent to be applied in a single SPE procedure. Furthermore, it is important to refer the importance of its application over methanol or acetonitrile (more commonly used), because ethanol is a "greener" solvent, the environmental effects resulting from the application of toxic solvents, such as methanol and acetonitrile, being avoided [164].

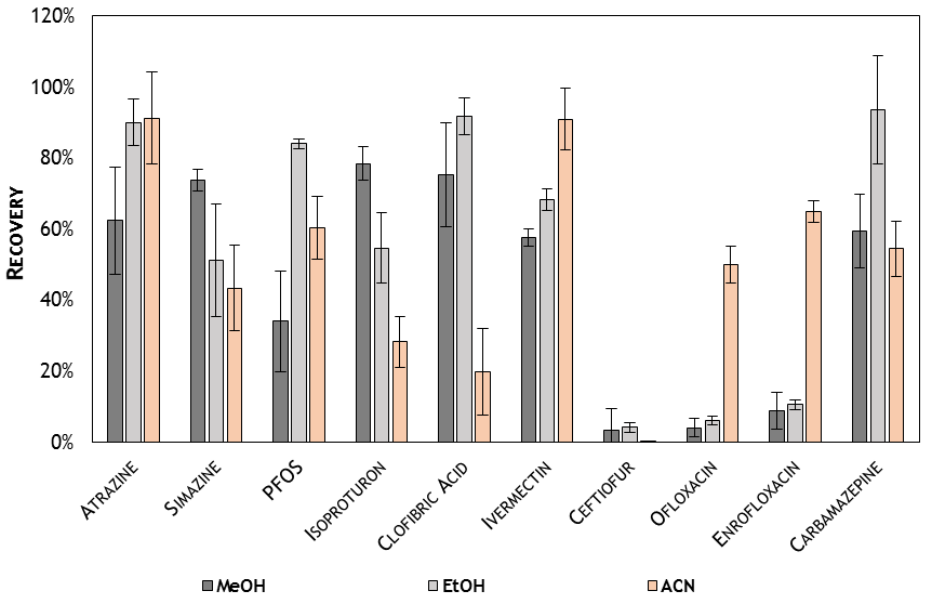


Figure 24. Recoveries obtained for 10 MPs, using different solvents (methanol, ethanol or acetonitrile) to extract 250 mL of spiked water samples (pH 3) through Oasis® HLB.

4.1.1.3. Type of cartridge

Considering the importance of the sorbent in the recovery of organic compounds, after optimizing the universal sorbent Oasis® HLB for acidic, neutral and basic compounds using ethanol and sample pH 3, two other cartridge types were tested: Oasis® MCX more suitable for extraction of basic compounds and Oasis® MAX which is more adequate for extraction of acidic compounds. However, Oasis® HLB cartridges provided higher recoveries for most compounds (data not shown). This finding was expected due to the different physical-chemical characteristics of the analytes under study, which included a wide range of p*K*a. Oasis® MAX and MCX cartridges are more suited for acidic and basic compounds, respectively, and for this reason compounds with lower p*K*a values, such as ofloxacin, presented better

recoveries with MAX, while compounds with higher p*K*a values, such as carbamazepine, had higher recoveries with MCX. In order to use a single SPE procedure, Oasis® HLB cartridges were chosen to extract and clean up the water samples in the present study.

4.1.1.4. Sample volume

Different sample volumes (250, 500 and 1000 mL) were tested to evaluate the breakthrough volume. *Figure 25* shows the recoveries obtained for the studied compounds, varying the volume of sample percolating through the cartridge.

The sample volume of 500 mL resulted in the highest recoveries for the majority of compounds and therefore, it was selected as the optimum volume. According to the overall results, Oasis® HLB cartridges, ethanol as solvent and 500 mL of water samples acidified to pH 3, were the conditions that provided higher recoveries for most compounds, these conditions being chosen for extraction of the MPs from water samples. The other group of MPs (selected from the Watch List) were also extracted using a similar SPE procedure that was already optimized in the laboratory for those specific compounds [166].

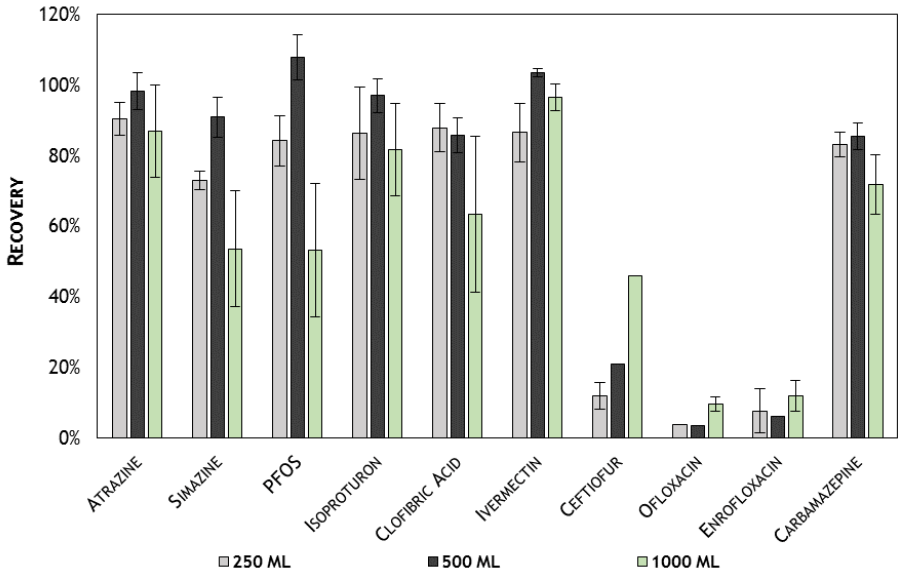


Figure 25. Recoveries obtained for 10 MPs, extracting different sample volumes (250, 500 and 1000 mL) of spiked water (pH 3) through Oasis® HLB cartridges and using ethanol as solvent.

4.1.2. UHPLC-MS/MS

4.1.2.1. Chromatographic separation

A Kinetex™ 1.7 µm XB-C18 100 Å column (100 × 2.1 mm, i.d.) was used to separate the analytes present in the reconstituted extracts. Its low diameter and length, as well as the sub-2 µm particles of the stationary phase, favour the chromatographic separation. In this work, different mobile phases were tested in order to improve resolution and the sensitivity of the veterinary drugs **(**ofloxacin, enrofloxacin, ceftiofur and ivermectin). The other compounds (PFOS, atrazine, simazine, isoproturon, clofibric acid and carbamazepine) [164] were included in an optimized analytical method developed in the laboratory

for 10 Watch List compounds (Method B) [166], using a mobile phase of methanol/water (75, 25, v/v) performed at gradient mode and flow rate of 0.25 mL min^{-1}.

For the 4 veterinary drugs, several combinations of organic and aqueous phases, as well as different gradients, were evaluated using methanol, ethanol or acetonitrile as organic phase and ultrapure water, ammonium acetate 10 mM or 0.1% of formic acid as aqueous phase. The main goal of the UHPLC optimization was to establish a suitable mobile phase for these 4 compounds (Method A). Despite the attempts to use ethanol as organic solvent, due to its "green characteristics", the combination of methanol and 0.1% formic acid aqueous solution (initial conditions:60/40, v/v) was the most adequate mobile phase for all compounds *(Appendix A4).* The flow rate and the gradient mode were optimized aiming to improve resolution and peak shape and to reduce the analysis time. The flow was set at 0.20 mL min^{-1} and the gradient was programmed as described in Section 3.2.2, with a total run time of 13 min. For the analyses, the temperature of the column oven and autosampler were set at 35 ºC and 4 ºC, respectively.

4.1.2.2. Mass spectrometry (MS/MS) conditions

The tandem MS technique was applied using a triple quadrupole mass spectrometer detector, which allows the quantification of MPs and their identity confirmation. The MS conditions for the set of MPs (veterinary drugs) were optimized. The precursor ion for each analyte was selected by injecting, directly, the individual standard solutions (10 mg L^{-1}) in full scan mode. All compounds showed higher response in positive mode of ionization (PI), being the molecular ion of each compound $[M+H \text{ or } M+Na]^+$ selected as precursor ion. All compounds presented two or more fragments of the precursor ion, being the SRM1 selected for their quantification *(Table 2).* The retention time and SMR2 were used for confirmation of their identity, analysing the ion ratio (SRM1/SMR2) values, as recommended in the European Commission Decision 2002/657/EC. Parameters such as decluttering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were also optimized, and the results obtained for each SRM of the individual compounds are summarized in *Table 2.* For this group of MPs, MS parameters were optimized, namely capillary voltage, drying gas flow, nebulizing gas flow, desolvation temperature and source temperature and the best results were obtained at 4.5 kV, 10 dm^3 min^{-1}, 3 dm^3 min^{-1}, 250 ºC and 450 ºC, respectively *(Appendix A5).* Appendix A6 summarizes the optimized MS conditions for the other MPs analyzed (Watch List compounds, PSs, clofibric acid and carbamazepine).

Table 2. Optimized MS parameters for SRM analysis of the target analytes under positive ionization mode.

Class and sub-class	Analyte	Retention Time (min)	Precursor ion (m/z)	Quantification (SRM [a])				Identification (SRM [a])				Ion ratio (±SD)
				Product Ion (m/z)	DP[b] (V)	CE[c] (V)	CXP[d] (V)	Product Ion (m/z)	DP[b] (V)	CE[c] (V)	CXP[d] (V)	
PHARMACEUTICALS												
	Ofloxacin-d3 (internal standard)	1.46	365.1	321.2	-18	-20	-21	-	-	-	-	n.a [e]
	Ofloxacin	1.46	361.9	318.2	-28	-19	-21	261.1	-28	-29	-26	1.17 (±0.23)
Veterinary drugs	Enrofloxacin	1.48	360.2	316.2	-17	-21	-21	342.2	-17	-23	-23	1.62 (±0.32)
	Ceftiofur	2.13	524.1	241.0	-26	-19	-25	210.1	-26	-24	-20	2.66 (±0.53)
	Ivermectin	8.03	897.5	753.3	-26	-40	-22	183.2	-26	-49	-10	1.38 (±0.28)

[a] SRM selected reaction monitoring.

[b] DP is the declustering potential.

[c] CE is the collision energy.

[d] CXP is the collision cell exit potential.

[e] n.a. is not applicable.

4.1.2.3. Method validation

Two SPE-UHPLC-MS/MS analytical methods were validated in accordance with the international criteria [167] and works published elsewhere [168-170]: (i) the SPE-UHPLC-MS/MS to analyse 4 veterinary drugs (method A); (ii) the previously developed method to analyse 10 Watch List compounds was revalidated to include the 4 PSs, clofibric acid and carbamazepine (Method B). *Table 3* shows the results obtained for linearity and range and IDL, IQL, MDL, MQL, whereas *Table 4* describes recovery, accuracy, intra and inter-batch precision values. Appendix A7 summarizes the values of these parameters for the compounds not optimized in this study (i.e., Watch List compounds).

Table 3. Range, linearity, instrument and method detection and quantification limits for 10 target compounds (4 PSs, clofibric acid, carbamazepine, 4 veterinary drugs).

Class and sub-class	Analyte	Range (ng L^{-1})	r^2	IDL[a] (µg L^{-1})	IQL[b] (µg L^{-1})	MDL[c] (ng L^{-1})	MQL[d] (ng L^{-1})
Industrial Compound	PFOS	1-100	0.9958	0.31	0.95	0.16	0.48
Pesticides							
Triazine	Atrazine	10-400	0.9938	0.14	0.44	0.07	0.22
	Simazine	10-400	0.9981	1.24	3.76	0.62	1.88
Phenylurea	Isoproturon	1-100	0.9995	0.44	1.33	0.22	0.67
Herbicide	Clofibric Acid	1-100	0.9965	0.31	0.93	0.15	0.47
Pharmaceuticals							
Psychiatric drug	Carbamazepine	5-100	0.9934	0.04	0.12	0.62	1.88
	Ofloxacin	4-400	0.9958	1.23	3.74	0.62	1.87
Veterinary drugs	Enrofloxacin	10-400	0.9953	0.33	1.01	0.17	0.50
	Ceftiofur	4-400	0.9945	0.26	0.78	0.13	0.39
	Ivermectin	4-400	0.9997	1.50	4.54	0.75	2.27

[a] is instrument detection limit.

[b] is instrument quantification limit.

[c] is method detection limit .

[d] is method quantification limit.

Table 4. Recovery, accuracy and precision (intra- and inter-batch) for 10 target compounds (4 PSs, clofibric acid, carbamazepine, 4 veterinary drugs).

Class and sub-class	Analyte	Recovery (%)	Accuracy (%)	Intra-batch precision (RSD)	Inter-batch precision (RSD)
Industrial Compound	PFOS	107.77±6.36	89.66±14.32	<14.9	<17.8
Pesticides					
Triazine	Atrazine	98.10±5.25	93.93±11.89	<13.5	<15.2
	Simazine	90.81±5.57	83.17±7.91	<9.9	<8.1
Phenylurea	Isoproturon	96.91±4.67	80.47±4.70	<6.8	<5.8
Herbicide	Clofibric Acid	85.75±4.90	116.26±10.51	<19.3	<17.1
PHARMACEUTICALS					
Psychiatric drug	Carbamazepine	85.40±3.75	89.58±26.72	<7.7	<13.8
Veterinary drugs	Ofloxacin	3.67±0.00	93.89±28.80	<13.6	<11.6
	Enrofloxacin	6.20±0.00	111.40±9.16	<10.5	<12.3
	Ceftiofur	20.93±0.00	82.75±5.05	<13.0	<9.0
	Ivermectin	103.52±1.18	89.27±9.22	<11.7	<13.4

The calibration curves for each MP (*Table 3*) were drawn by the internal standard calibration method. Since labelled standards for all MPs are not available, different sets of analytes were defined to relate with the respective internal standard (ofloxacin-d3 was used for veterinary drugs, atrazine-d5 for pesticides and the industrial compound, azithromicyn-d3 for carbamazepine), as performed in other studies [164]. The coefficients of determination (r^2) for all compounds were between 0.9934 and 0.9997. The ranges of MDL and MQL were 0.03-0.75 ng L^{-1} and 0.09-2.27 ng L^{-1}, respectively, enabling to detect the target MPs at residual concentrations.

Recoveries values higher than 85% were obtained for all compounds (*Table 4*), except for ceftiofur (20.93%), enrofloxacin (6.20%) and ofloxacin (3.67%). Despite the poor recovery observed for these 3 MPs, the method was fully validated and these compounds were included due to the high reproducibility of the results and the use of internal standard calibration. The different recoveries verified among analytes are due to their wide variable chemistry nature and individual physical-chemical characteristics that interfer in the SPE. The accuracy varied from 81% to 116%, which is within the range of ± 20% of the nominal concentration, in accordance with the international guidelines (80-120%) [167]. Relative

standard deviation (RSD) of the replicate analyses was used to express the precision of the method and complyed with the international criteria suggesting an agreement of the results when RSD is lower than 15% between the different QCs with the same concentration (or 20% for the lower concentration QC) [167].

4.1.2.4. Matrix effect

The matrix effect in the ionization process may result in the suppression or enhancement of the analytes ionization, which consequently leads to the decrease or increase of the signal, respectively. Thus, it is important to determine the matrix effect, in order to avoid erroneous interpretation of results. *Table 5* shows the matrix effect values of the compounds for which the SPE-UHPLC-MS/MS method was fully optimized.

Table 5. Matrix effect of the veterinary drugs compounds.

Class and sub-class	Analyte	Matrix effect (%)
PHARMACEUTICALS		
Veterinary drugs	Ofloxacin	87.12
	Enrofloxacin	63.22
	Ceftiofur	67.36
	Ivermectin	77.98

The matrix effect ranged between 63.22% and 87.12%, demonstrating that signal suppression occurs in the ionization source for the 4 pharmaceuticals. In fact, the signal suppression is more frequently observed than the signal enhancement [20]. The matrix effect by itself does not justify the poor recovery obtained for these set of compounds, the low efficiency on the SPE extraction also playing a role. For ivermectin (recovery near 100%), the matrix effect was not expected; however, a signal suppression was observed. Thus, in the future, recovery and matrix effect assays have to be repeated for this particular antihelminthic, in order to check the obtained results.

4.2. Aquaculture effluent

4.2.1. Quantification of MPs

As referred before, the water samples collected in March and May (in 2016) from a freshwater fish farm, were analysed using two SPE-UHPLC-MS/MS methods, one fully optimized and validated in the present work (Method A), and another already developed in the same laboratory and that was now revalidated (Method B) [164, 166]. The results obtained are illustrated in *Figure 26*.

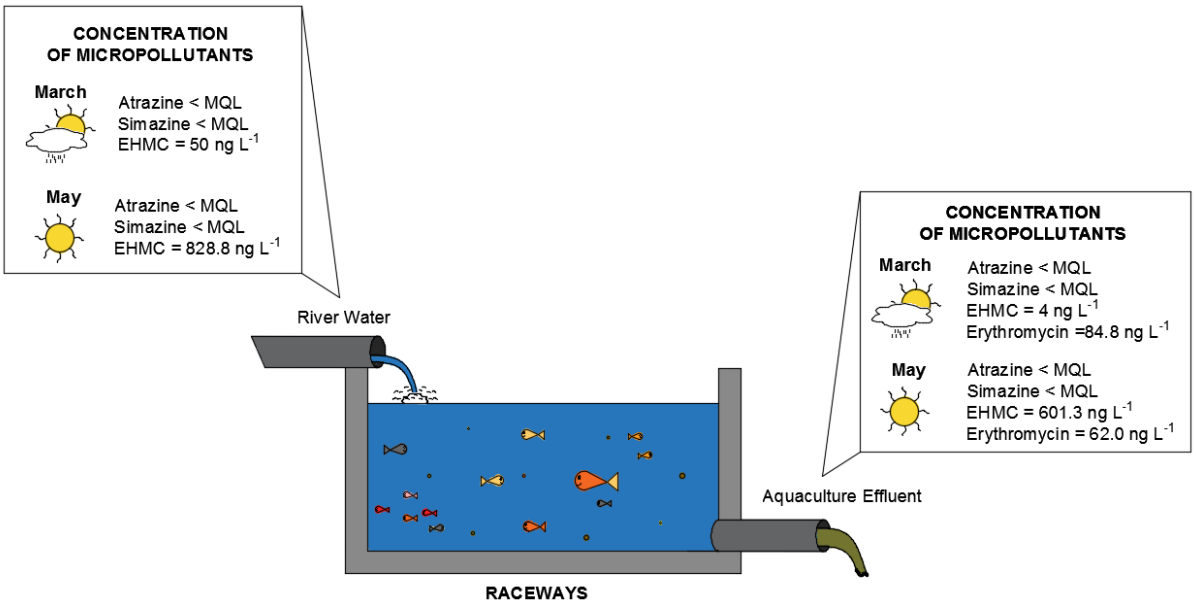


Figure 26. Concentration of MPs (ng L^{-1}) at inlet and outlet of aquaculture farm.

In both campaigns, only 3 and 4 MPs (out of 21 analysed) were detected in the aquaculture influent and effluent **(***Figure 26***)**, respectively. In both inlet and outlet samples, the PSs atrazine and simazine were detected under their MQL, whereas EHMC was quantified and its concentration reduced from the influent consisting in river water (50.0 ng L^{-1} in March and 828.8 ng L^{-1} in May) to the effluent (4.0 ng L^{-1} in March and 601.3 ng L^{-1} in May). Erythromycin was only detected in the effluent (84.8 ng L^{-1} in March and 62.0 ng L^{-1} in May). The different levels of concentration verified for EHMC in the two sampling campaigns (March and May) are notorious. Several factors can contribute for this ambiguous occurrence, namely weather conditions and human activities. The sampling campaigns were performed in March (day 21) and May (day 23), periods with a very dissimilar weather conditions, e.g., in May the average air temperature was quite higher than in March, and the week before collecting the samples in March was rainy [173]. The different weather conditions might explain the lower concentration of EHMC that was found in the first sampling campaign. EHMC is an organic UV filter used in personal care products, such as sunscreens, beauty creams, hair sprays, shampoos, among others. Nevertheless, sunscreens are also applied in many industrial products, e.g. paints, plastics and textile materials, in order to prevent degradation of polymers and pigments [174, 175]. There are many industries near the aquaculture farm, namely timber, plastic, personal hygiene, fragrance and cosmetics industries. Therefore, sporadic discharges after the first collection might also justify the higher concentration values of EHMC that were found in May. The analysis of aquaculture water inflow and outflow allowed to conclude that erythromycin was only quantifiable in water samples collected after the fish farming, but at extremely low concentrations (< 100 ng L^{-1}), much lower than those found for other compounds in surface water or even drinking water. In fact, erythromycin is an antibiotic used in aquaculture against the Gram-positive cocci, one major concern for trout framing [176], explaining its detection in fish farms The other veterinary drugs, known as commonly used for aquaculture purposes, as well as the other organic compounds (some PSs, 10 Watch List compounds, carbamazepine and clofibric acid) were not detected.

4.2.2. Other analysis

In addition to organic MPs quantification, the dissolved organic carbon (DOC) and ions were also analysed. The obtained results are shown in *Figure 27.*

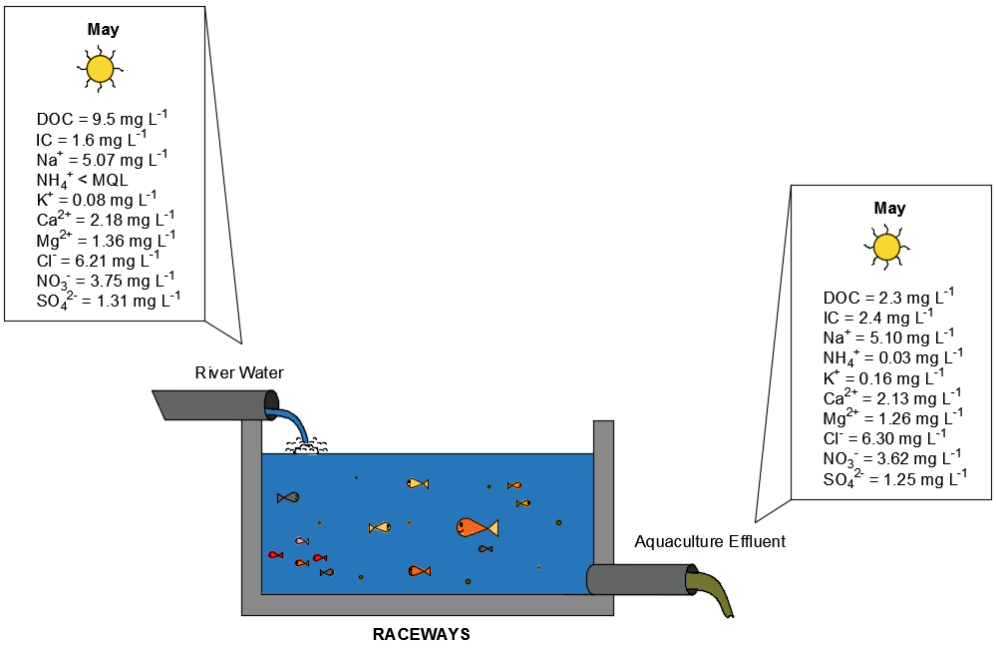


Figure 27. DOC and ions concentrations (mg L^{-1}) at inlet and outlet of aquaculture farm.

DOC decreased from the aquaculture influent (9.5 mg L^{-1}) to the effluent (2.3 mg L^{-1}), whereas the opposite trend was verified for IC, which increased from 1.6 mg L^{-1} to 2.4 mg L^{-1}. Regarding the ions concentration, there was no significant difference between the inlet and outlet of the aquaculture farm. The cations varied from lower than MQL (ammonium) to 5.07 mg L^{-1} (sodium) in the inlet and from 0.03 mg L^{-1} (ammonium) to 5.10 mg L^{-1} (sodium) in the outlet. For anions, sulphate was detected at the lowest concentrations (1.31 mg L^{-1} in the influent and 1.25 mg L^{-1} in the effluent) and chloride was the anion quantified at the highest concentrations (6.21 mg L^{-1} in the inlet and 6.30 mg L^{-1} at the outlet).

4.3. Treatment of aquaculture effluents

4.3.1. CWs experiments

4.3.1.1. Non-spiked effluents

Aquaculture effluent samples collected in the second sampling (May 2016), were submitted to treatment in CW systems (CW_1 1.5 L) during one week, to assess the removal of MPs previously detected in the effluents *(Figure 28).*

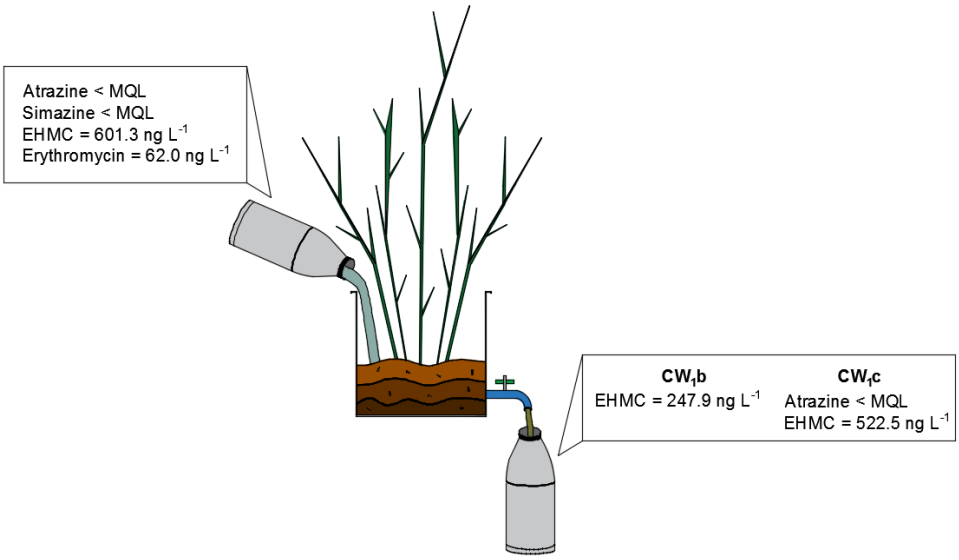


Figure 28. Concentrations of MPs (ng L^{-1}) in non-spiked aquaculture effluent before and after treatment in CW systems.

Three CW_1 replicates (a, b, c) were assembled. Although the experiments were carried out in triplicate, CW_1a was not here considered, since its performance was very different from the others. The CW_1a was placed in a slightly different local and this fact might have affected several parameters such as the light and consequently the performance. The results obtained for the two other systems, CW_1b and CW_1c, were comparable and showed the ability of CWs to remove simazine (pesticide) and erythromycin (antibiotic), which were not detected in the CWs treated effluent. Atrazine (pesticide) was detected under its MQL in the inlet of both CW_1 and also in the outlet of CW_1c, but not after treatment in CW_1b. Both simazine and atrazine were present in very low concentrations, being difficult to evaluate the efficiency of the process. EHMC was detected at 248 ng L^{-1} (CW_1b) and 523 ng L^{-1} (CW_2c), corresponding to EHMC removals of 56% and 13%, respectively. The dissimilar results obtained for EHMC may be explained by the inherent heterogeneity when performing biological-based experiments. There are no studies in literature with this pollutant and CWs *(Figure 10)* and thus, it is not possible to compare the results obtained in the present work with those already published. For erythromycin, CWs demonstrated a good performance *(Figure 28)*, a complete elimination (under MDL) being achieved, similarly to other published studies dealing with CWs [130]. Water samples resulting from CWs were also analysed for determination of DOC and ions concentration *(Figure 29)*.

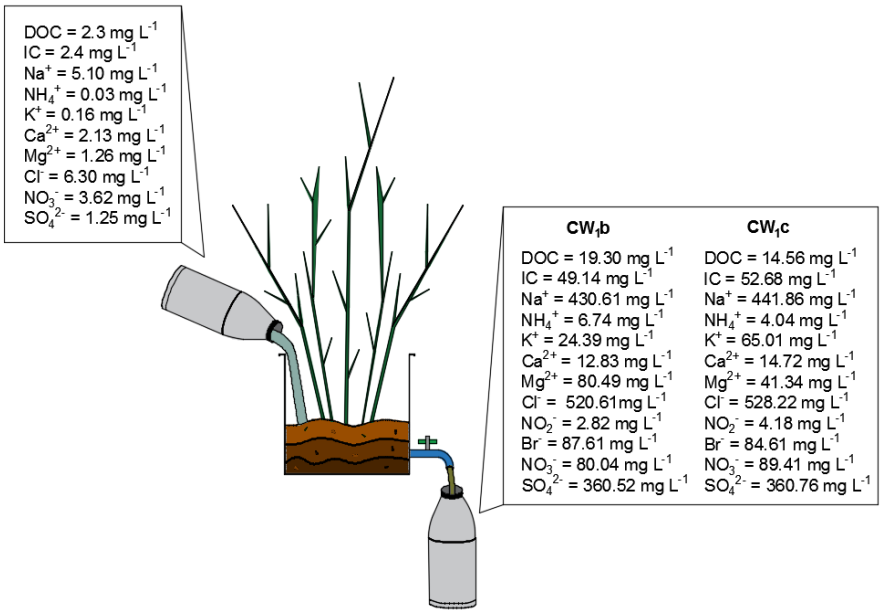


Figure 29. DOC and ions concentrations (mg L^{-1}) in non-spiked aquaculture effluent before and after treatment in CW systems.

Results revealed an increased concentration of DOC after treatment, from 2.3 mg L^{-1} (initial concentration) to 19.30 mg L^{-1} and 14.56 mg L^{-1} in CW_1b and CW_1c, respectively. This fact can be partially explained by the nutrient solution added previously to these CWs (total volume of 6 L), which contains the organic compound EDTA. Furthermore, the biological systems contain other organic substances, namely in the substrate, such as humic acids, that can also contribute to this increase. The IC increased by 25-fold in both CWs and the same behaviour was found for the concentration of ions. Some ions species, such as nitrite and bromide anions, were only detected after treatment in CWs. All these findings are probably a consequence of the addition of the nutrients solution and the release of IC and/or ions from the sediments and/or plants.

4.3.1.2. Spiked effluents

In addition to the non-spiked experiments, the same aquaculture effluent samples collected in May, were spiked with a set of 10 MPs at 100 ng L^{-1}, namely atrazine, simazine, erythromycin, EHMC, clofibric acid, carbamazepine, ofloxacin, enrofloxacin, ceftiofur and ivermectin and treated by CWs (CW_2). The choice of this group of target compounds was based on the inclusion of the MPs found in the non-spiked effluent (atrazine, simazine, erythromycin and EHMC), two known recalcitrant compounds in the environment (clofibric acid and carbamazepine) and pharmaceuticals commonly used in fish farming (ofloxacin, enrofloxacin, ceftiofur and ivermectin). Thus, organic PSs, CECs of the Watch List and other compounds with interest in this study, due to their recalcitrance and/or known usage, were encompassed.

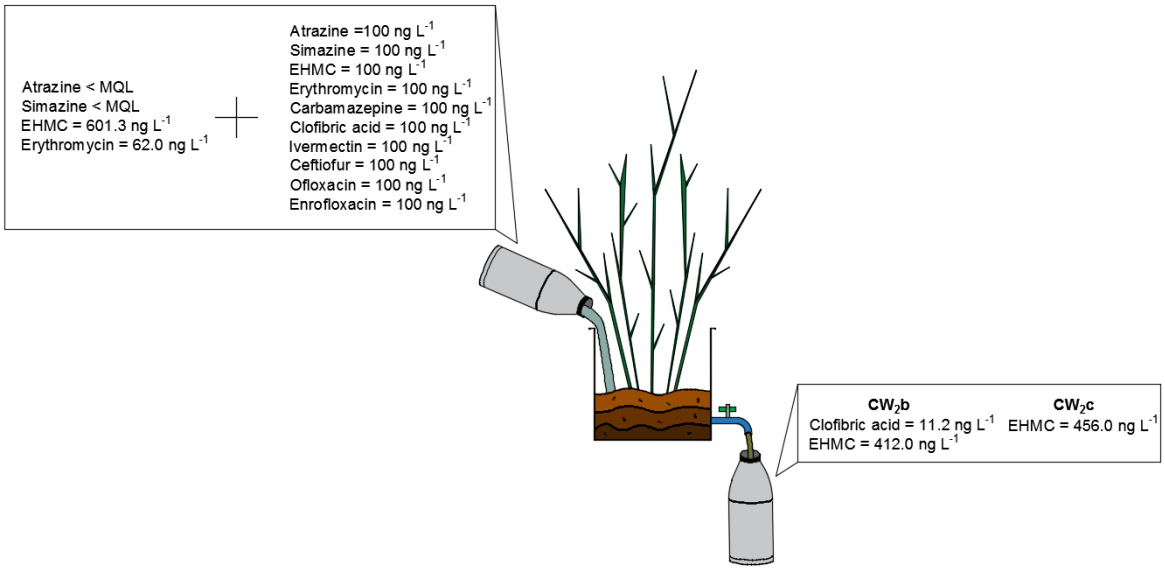


Figure 30. Concentration of MPs (ng L^{-1}) in spiked-aquaculture effluent before and after treatment in CWs systems.

The overall results (*Figure 30)* showed that, CW_2 systems were able to remove the pharmaceuticals and pesticides, which were not detected in the collected treated effluent. Once again only two CWs replicates were considered. Some studies described that CWs, in general, are not able to completely remove carbamazepine; however, other studies refer that a high removal can be achieved, depending on the conditions involved in the treatment [177, 178], which vary according to the different studies. The pharmaceutical erythromycin and the veterinary drugs, frequently reported in aquaculture effluents, were also removed by the CW_2 systems, emphasizing the possible application of this type of treatment for aquaculture effluents. Other studies have demonstrated that CWs can also efficiently remove organic matter, nitrogen and phosphorus, among others, in aquaculture effluents [179], but studies related with CWs to remove organic MPs in aquaculture effluents are missing in literature. Atrazine and simazine were totally removed by the two CW_2 systems, as observed in the non-spiked experiment. Regarding these pesticides, the complete removal was only reported before for atrazine and the removal achieved depends on the characteristics of the studies *(Figure 9).* The available studies focused in simazine and applying other wetland configurations, did not show a complete removal of this pesticide [85, 87, 89] but higher concentrations than those used in this work, were used [54, 87]. Therefore, the good performance to remove simazine is described for the first time. EHMC was not totally removed in the CW experiments (*Figure 30*), as occurred when using non-spiked samples (*Figure 28).* Clofibric acid (a known recalcitrant compound in the environment) was removed by 89% and 100% for CW_2b and CW_2c, respectively. In general, wetland systems showed a good performance for the MPs under analysis, except for EHMC.

Figure 31 shows the DOC and ions concentrations for the spiked aquaculture effluents before and after treatment.

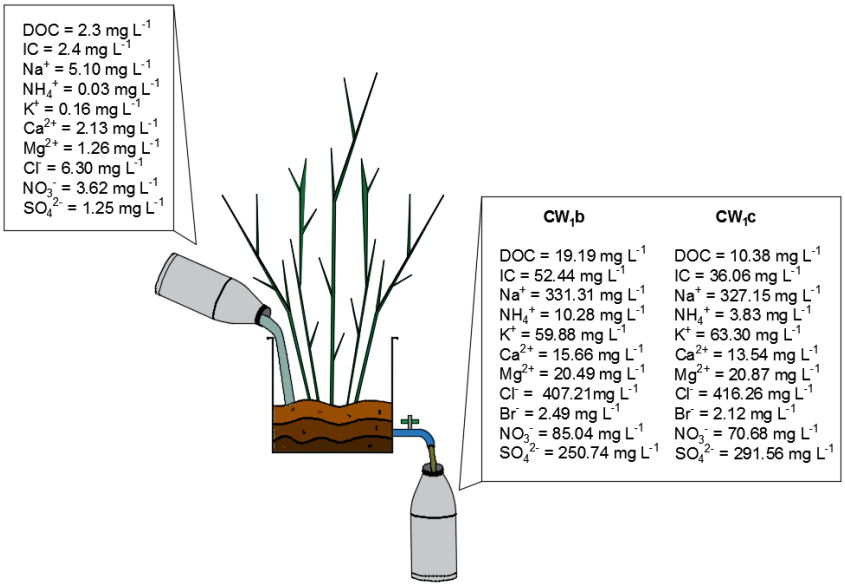


Figure 31. Concentration of DOC and ions (mg L^{-1}) in spiked-aquaculture effluent before and after treatment in CWs systems.

The obtained results for CW_2 systems are in accordance with the results observed for the non-spiked effluent experiments (CW_1) and might be justified by the same reasons referred in Section 4.3.1.1. However, it is important to mention that in most studies employing CWs, it was verified a reduction of the organic matter and nutrients concentrations [180], although these compounds were in much higher concentrations in the influents. In general, organisms break down organic matter in order to produce new biomass, reproduce and sustain life, leading to the decrease of the organic content [181]. Removal of nutrients can occur by the uptake of plants and sediments in the case of phosphorus and nitrogen, and by nitrification/denitrification for nitrogen. All these processes are affected by several factors that originate different removal rates [180]. In the case of the present study, the concentration of organic matter and nutrients increased, although to levels still very low and below levels of concern. CWs revealing to be a good alternative for the removal MPs.

4.3.2. Lab-scale ozonation experiments

The water samples collected from CWs were then treated by ozonation during 10 min. The results obtained are shown in *Figures 32* and *33* for the sequence of non-spiked and spiked aquaculture effluents experiments, respectively.

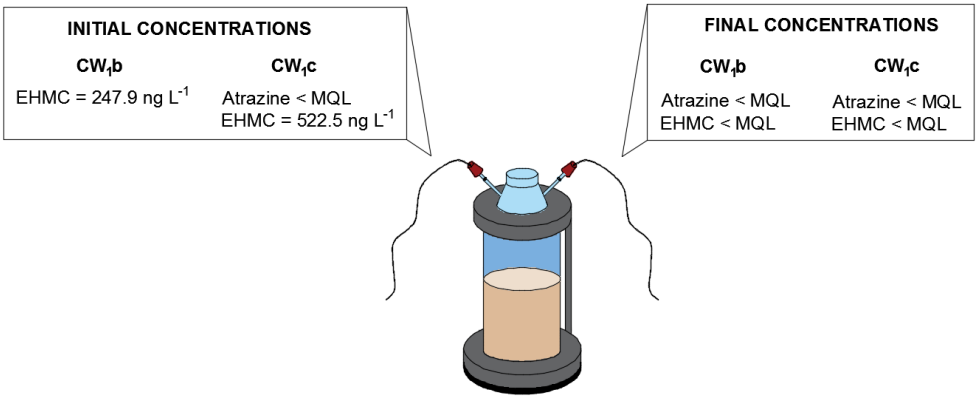


Figure 32. Concentrations of MPs (ng L^{-1}) in CWs treated non-spiked aquaculture effluents before and after ozonation.

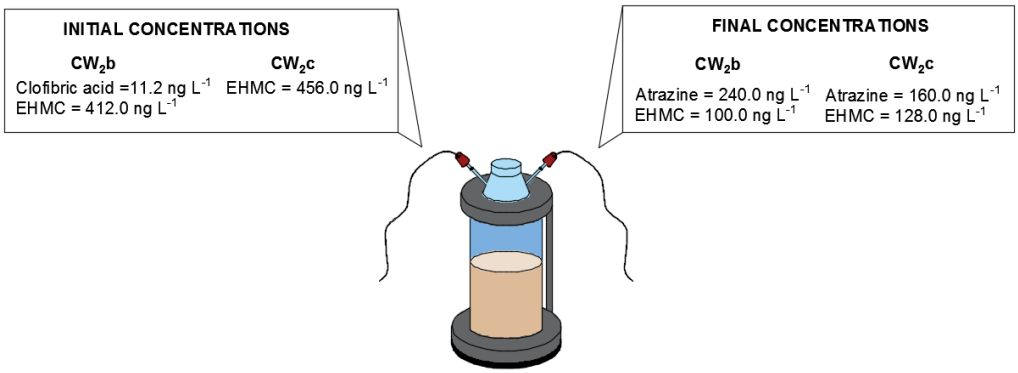


Figure 33. Concentrations of MPs (ng L^{-1}) in CWs treated spiked aquaculture effluents before and after ozonation.

Clofibric acid (herbicide) supplied in the spiked effluent and found after treatment in one CW experiment (CW_2b), was completely removed by ozonation. EHMC (organic UV filter) was found under MQL after ozonation in the non-spiked experiments (removal $\geq$ 98.5%) and 74% of this MP (on average) was removed in spiked experiments. Atrazine was not removed by ozonation in non-spiked or spiked effluents experiments, being even quantified after ozonation in the spiked effluents experiments. This fact is possibly related to sample contamination, occurring due to the use of atrazine in other experiments at higher concentrations (mg L^{-1} levels). In conclusion, the ozonation treatment was efficient to remove the target MPs and the elimination rate of EHMC can be improved in future experiments, by extending the reaction time.

Regarding the DOC and ions concentrations, no significant variations were observed before and after ozonation (data not shown).

5
Conclusions

The relevant information on the application of CWs for the removal of organic PSs (Directive 2013/39/EU) and/or CECs of the Watch List (Decision 2015/495/EU) was revised in a literature survey.

A SPE-UHPLC-MS/MS method (A) was fully optimized and validated to assess the occurrence of 4 veterinary pharmaceuticals (ceftiofur, ofloxacin, enrofloxacin and ivermectin) commonly applied for aquaculture practices in surface water, other SPE-UHPLC-MS/MS method (B), previously developed in the LSRE-LCM laboratory, was adapted and revalidated to analyse, in the same water matrix, some PSs of the Directive 2013/39/EU (3 pesticides and 1 industrial compound), 2 recalcitrant compounds frequently found in surface water (carbamazepine and acid clofibric) and 11 CECs of the first Watch List of Decision 2015/495/EU (6 pesticides, 4 pharmaceuticals and 1 organic UV filter). An eco-friendly solvent (ethanol) was selected in the optimization of SPE, minimizing the environmental impact of the sample preparation procedure. A Kinetex column and methanol/0.1% formic acid as mobile phase in gradient mode were used in the UHPLC-MS/MS method to analyse the 4 veterinary drugs, with a run time of 13 min. A single SPE procedure to extract all target compounds included in both UHPLC-MS/MS methods was optimized. The validation was performed according to the international guidelines and the results obtained for all parameters were in accordance with the standardized values.

The application of the methods to the analysis of freshwater aquaculture inlet and outlet samples, collected in a trout farm located in Portugal (March and May, 2016), showed the presence of 2 PSs (atrazine and simazine) and 2 Watch List CECs (EHMC and erythromycin) at ng L^{-1} levels. However, erythromycin, an antibiotic commonly administered to fishes, was only found in the outlet aquaculture water sample and at very low concentrations. Samples collected in March and May had a significant difference in the EHMC concentration, which was higher in May and can be related to the different weather conditions, or eventually to industrial discharges of effluents to the river between the sampling campaigns.

A set of CWs experiments was carried out in order to evaluate the performance of these systems to remove the MPs found in the aquaculture effluents (non-spiked), and another set of tests was performed with 10 MPs spiked at 100 ng L^{-1} in the same aquaculture effluents, including the 4 detected compounds, 4 veterinary drugs and 2 selected recalcitrant compounds (carbamazepine and clofibric acid). In general, the CWs systems demonstrated a good performance to remove all target compounds with exception of EHMC (Watch List). Short ozonation experiments demonstrated good efficiencies to oxidize the MPs not fully eliminated by CWs. Thus, the coupled CW-ozonation treatment was proved to be a good alternative for the removal of MPs, but more bench-scale research on this subject should be addressed for future applications at pilot-scale. Low-cost, by using CWs to remove a significant fraction of MPs, and high efficiency, by using ozonation to remove the remaining MPs, are expected by following this strategy that allows lower installation and operating ozonation costs than when this process is applied alone.

6
Future work

In this work, CW coupled to ozonation showed to be an interesting solution to remove the target MPs that were quantified by advanced analytical techniques. Other tasks may be addressed in the future, extending the research in this area that is still poorly explored:

- To perform seasonal sampling campaigns in different freshwater aquaculture farms in order to evaluate the most frequent MPs in this type of effluents;
- To perform CWs experiments using other conditions, in order to assess the effect of light, the minimum residence time needed to achieve the best performance as well as using effluents spiked with other MPs to analyse the treatment efficiency to remove a wider set of MPs;
- To encompass the removal of nutrients and organic matter from effluents, beyond the removal of MPs;
- To optimize the ozonation treatment, improving the efficiency of the coupled CW-ozonation process.

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7
Appendix

A1: List of priority substances in the field of water policy

Table A1. PSs defined in the Directive 2013/39/EU [10].

Number	CAS number (1)	EU number (2)	Name of priority substance (3)	Identified as priority hazardous substance
(1)	15972-60-8	240-110-8	Alachlor	
(2)	120-12-7	204-371-1	Anthracene	X
(3)	1912-24-9	217-617-8	Atrazine	
(4)	71-43-2	200-753-7	Benzene	
(5)	not applicable	not applicable	Brominated diphenylethers	X (4)
(6)	7440-43-9	231-152-8	Cadmium and its compounds	X
(7)	85535-84-8	287-476-5	Chloroalkanes, C_{10-13}	X
(8)	470-90-6	207-432-0	Chlorfenvinphos	
(9)	2921-88-2	220-864-4	Chlorpyrifos (Chlorpyrifos-ethyl)	
(10)	107-06-2	203-458-1	1,2-dichloroethane	
(11)	75-09-2	200-838-9	Dichloromethane	
(12)	117-81-7	204-211-0	Di(2-ethylhexyl)phthalate (DEHP)	X
(13)	330-54-1	206-354-4	Diuron	
(14)	115-29-7	204-079-4	Endosulfan	X
(15)	206-44-0	205-912-4	Fluoranthene	

Table A1. Continued

(16)	118-74-1	204-273-9	Hexachlorobenzene	X
(17)	87-68-3	201-765-5	Hexachlorobutadiene	X
(18)	608-73-1	210-168-9	Hexachlorocyclohexane	X
(19)	34123-59-6	251-835-4	Isoproturon	
(20)	7439-92-1	231-100-4	Lead and its compounds	
(21)	7439-97-6	231-106-7	Mercury and its compounds	X
(22)	91-20-3	202-049-5	Naphthalene	
(23)	7440-02-0	231-111-4	Nickel and its compounds	
(24)	not applicable	not applicable	Nonylphenols	X (5)
(25)	not applicable	not applicable	Octylphenols (6)	
(26)	608-93-5	210-172-0	Pentachlorobenzene	X
(27)	87-86-5	201-778-6	Pentachlorophenol	
(28)	not applicable	not applicable	Polyaromatic hydrocarbons (PAH) (7)	X
(29)	122-34-9	204-535-2	Simazine	
(30)	not applicable	not applicable	Tributyltin compounds	X (8)
(31)	12002-48-1	234-413-4	Trichlorobenzenes	
(32)	67-66-3	200-663-8	Trichloromethane (chloroform)	
(33)	1582-09-8	216-428-8	Trifluralin	X
(34)	115-32-2	204-082-0	Dicofol	X
(35)	1763-23-1	217-179-8	Perfluorooctane sulfonic acid and its derivatives (PFOS)	X
(36)	124495-18-7	not applicable	Quinoxyfen	X
(37)	not applicable	not applicable	Dioxins and dioxin-like compounds	X (9)
(38)	74070-46-5	277-704-1	Aclonifen	
(39)	42576-02-3	255-894-7	Bifenox	
(40)	28159-98-0	248-872-3	Cybutryne	
(41)	52315-07-8	257-842-9	Cypermethrin (10)	
(42)	62-73-7	200-547-7	Dichlorvos	
(43)	not applicable	not applicable	Hexabromocyclododecanes (HBCDD)	X (11)
(44)	76-44-8/ 1024-57-3	200-962-3/ 213-831-0	Heptachlor and heptachlor epoxide	X
(45)	886-50-0	212-950-5	Terbutryn	

(1) CAS: Chemical Abstracts Service.

([2]) EU-number: European Inventory of Existing Commercial Substances (EINECS) or European List of Notified Chemical Substances (ELINCS).

([3]) Where groups of substances have been selected, unless explicitly noted, typical individual representatives are defined in the context of the setting of environmental quality standards.

([4]) Only Tetra, Penta, Hexa and Heptabromodiphenylether (CAS -numbers 40088-47-9, 32534-81-9, 36483-60-0, 68928-80-3, respectively).

([5]) Nonylphenol (CAS 25154-52-3, EU 246-672-0) including isomers 4-nonylphenol (CAS 104-40-5, EU 203-199-4) and 4- nonylphenol (branched) (CAS 84852-15-3, EU 284-325-5).

([6]) Octylphenol (CAS 1806-26-4, EU 217-302-5) including isomer 4-(1,1',3,3'-tetramethylbutyl)-phenol (CAS 140-66-9, EU 205-426-2).

([7]) Including benzo(a)pyrene (CAS 50-32-8, EU 200-028-5), benzo(b)fluoranthene (CAS 205-99-2, EU 205-911-9), benzo(g,h,i)perylene (CAS 191-24-2, EU 205-883-8), benzo(k)fluoranthene (CAS 207-08-9, EU 205-916-6), indeno(1,2,3-cd)pyrene (CAS 193-39-5, EU 205-893-2) and excluding anthracene, fluoranthene and naphthalene, which are listed separately.

([8]) Including tributyltin-cation (CAS 36643-28-4).

([9]) This refers to the following compounds: 7 polychlorinated dibenzo-p-dioxins (PCDDs): 2,3,7,8-T4CDD (CAS 1746-01-6), 1,2,3,7,8-P5CDD (CAS 40321-76-4), 1,2,3,4,7,8- H6CDD (CAS 39227-28-6), 1,2,3,6,7,8-H6CDD (CAS 57653-85-7), 1,2,3,7,8,9-H6CDD (CAS 19408-74-3), 1,2,3,4,6,7,8-H7CDD (CAS 35822-46-9), 1,2,3,4,6,7,8,9-O8CDD (CAS 3268-87-9) 10 polychlorinated dibenzofurans (PCDFs): 2,3,7,8-T4CDF (CAS 51207-31-9), 1,2,3,7,8-P5CDF (CAS 57117-41-6), 2,3,4,7,8-P5CDF (CAS 57117-31-4), 1,2,3,4,7,8-H6CDF (CAS 70648-26-9), 1,2,3,6,7,8-H6CDF (CAS 57117-44-9), 1,2,3,7,8,9-H6CDF (CAS 72918- 21-9), 2,3,4,6,7,8-H6CDF (CAS 60851-34-5), 1,2,3,4,6,7,8-H7CDF (CAS 67562-39-4), 1,2,3,4,7,8,9-H7CDF (CAS 55673-89-7), 1,2,3,4,6,7,8,9-O8CDF (CAS 39001-02-0) 12 dioxin-like polychlorinated biphenyls (PCB-DL): 3,3',4,4'-T4CB (PCB 77, CAS 32598-13-3), 3,3',4',5-T4CB (PCB 81, CAS 70362- 50-4), 2,3,3',4,4'-P5CB (PCB 105, CAS 32598-14-4), 2,3,4,4',5-P5CB (PCB 114, CAS 74472-37-0), 2,3',4,4',5-P5CB (PCB 118, CAS 31508-00-6), 2,3',4,4',5'-P5CB (PCB 123, CAS 65510-44-3), 3,3',4,4',5-P5CB (PCB 126, CAS 57465-28-8), 2,3,3',4,4',5-H6CB (PCB 156, CAS 38380-08-4), 2,3,3',4,4',5'-H6CB (PCB 157, CAS 69782-90-7), 2,3',4,4',5,5'-H6CB (PCB 167, CAS 52663-72-6), 3,3',4,4',5,5'-H6CB (PCB 169, CAS 32774-16-6), 2,3,3',4,4',5,5'-H7CB (PCB 189, CAS 39635-31-9).

([10]) CAS 52315-07-8 refers to an isomer mixture of cypermethrin, alpha-cypermethrin (CAS 67375-30-8), beta-cypermethrin (CAS 65731-84-2), theta-cypermethrin (CAS 71697-59-1) and zeta-cypermethrin (52315-07-8).

([11]) This refers to 1,3,5,7,9,11-Hexabromocyclododecane (CAS 25637-99-4), 1,2,5,6,9,10- Hexabromocyclododecane (CAS 3194-55-6), α-Hexabromocyclododecane (CAS 134237-50-6), β-Hexabromocyclododecane (CAS 134237-51-7) and γ- Hexabromocyclododecane (CAS 134237-52-8).

A2: Watch List of substances for Union-wide monitoring in the field of water policy

Table A2. Watch List of substances for Union-wide monitoring in the field of water policy defined in the COMMISSION IMPLEMENTING DECISION (EU) 2015/495 of 20 March 2015 [11].

Name of substance/group of substances	CAS number (1)	EU number (2)	Indicative analytical method (3) (4) (5)	Maximum acceptable method detection limit (ng/l)
17-Alpha-ethinylestradiol (EE2)	57-63-6	200-342-2	Large-volume SPE — LC-MS-MS	0,035
17-Beta-estradiol (E2), Estrone (E1)	50-28-2, 53-16-7	200-023-8	SPE — LC-MS-MS	0,4
Diclofenac	15307-86-5	239-348-5	SPE — LC-MS-MS	10
2,6-Ditert-butyl-4-methylphenol	128-37-0	204-881-4	SPE — GC-MS	3 160
2-Ethylhexyl 4-methoxycinnamate	5466-77-3	226-775-7	SPE — LC-MS-MS or GC-MS	6 000
Macrolide antibiotics (6)			SPE — LC-MS-MS	90
Methiocarb	2032-65-7	217-991-2	SPE — LC-MS-MS or GC-MS	10
Neonicotinoids (7)			SPE — LC-MS-MS	9
Oxadiazon	19666-30-9	243-215-7	LLE/SPE — GC-MS	88
Tri-allate	2303-17-5	218-962-7	LLE/SPE — GC-MS or LC-MS-MS	670

(1) Chemical Abstracts Service.

(2) European Union number — not available for all substances.

(3) To ensure comparability of results from different Member States, all substances shall be monitored in whole water samples.

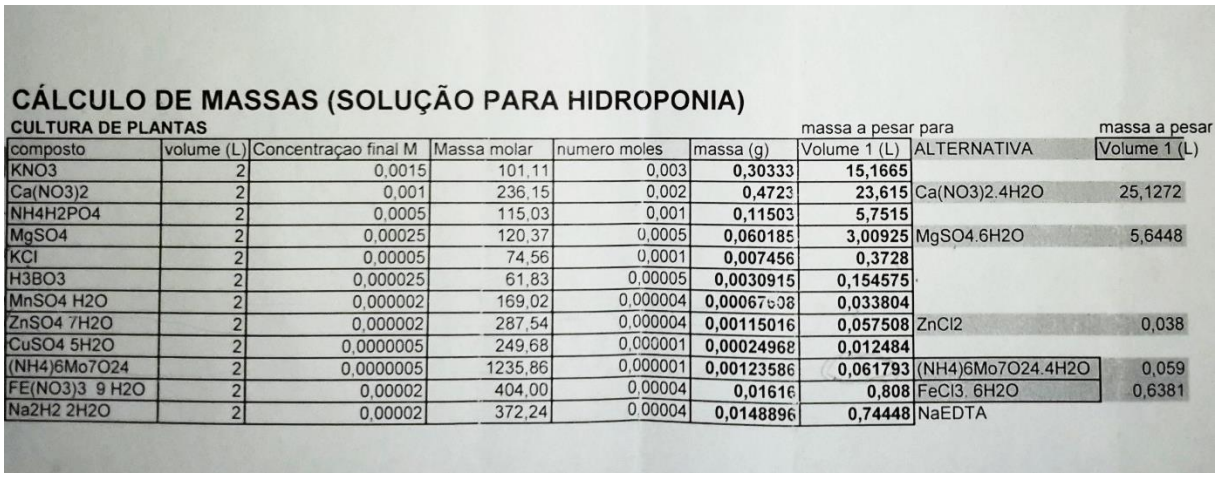
(4) Extraction methods: LLE — liquid liquid extraction, SPE — solid-phase extraction. Analytical methods: GC-MS — Gas chromatography-mass spectrometry, LC-MS-MS — Liquid chromatography (tandem) triple quadrupole mass spectrometry.

(5) For monitoring 2-Ethylhexyl 4-methoxycinnamate in suspended particulate matter (SPM) or in sediment (size < 63 μm), the following analytical method is indicated: SLE (solid liquid extraction) — GC-MS, with a maximum detection limit of 0,2 mg/kg.

(6) Erythromycin (CAS number 114-07-8, EU number 204-040-1), Clarithromycin (CAS number 81103-11-9), Azithromycin (CAS number 83905-01-5, EU number 617-500-5).

(7) Imidacloprid (CAS number 105827-78-9/138261-41-3, EU number 428-040-8), Thiacloprid (CAS number 111988-49-9), Thiamethoxam (CAS number 153719-23-4, EU number 428-650-4), Clothianidin (CAS number 210880-92-5, EU number 433-460-1), Acetamiprid (CAS number 135410-20-7/160430-64-8).

A3: Nutrient solution for CWs irrigation



CÁLCULO DE MASSAS (SOLUÇÃO PARA HIDROPONIA)

CULTURA DE PLANTAS

composto	volume (L)	Concentraçao final M	Massa molar	numero moles	massa (g)	massa a pesar para Volume 1 (L)	ALTERNATIVA	massa a pesar Volume 1 (L)
KNO3	2	0,0015	101,11	0,003	**0,30333**	**15,1665**		
Ca(NO3)2	2	0,001	236,15	0,002	**0,4723**	**23,615**	Ca(NO3)2.4H2O	25,1272
NH4H2PO4	2	0,0005	115,03	0,001	**0,11503**	**5,7515**		
MgSO4	2	0,00025	120,37	0,0005	**0,060185**	**3,00925**	MgSO4.6H2O	5,6448
KCl	2	0,00005	74,56	0,0001	**0,007456**	**0,3728**		
H3BO3	2	0,000025	61,83	0,00005	**0,0030915**	**0,154575**		
MnSO4 H2O	2	0,000002	169,02	0,000004	**0,00067608**	**0,033804**		
ZnSO4 7H2O	2	0,000002	287,54	0,000004	**0,00115016**	**0,057508**	ZnCl2	0,038
CuSO4 5H2O	2	0,0000005	249,68	0,000001	**0,00024968**	**0,012484**		
(NH4)6Mo7O24	2	0,0000005	1235,86	0,000001	**0,00123586**	**0,061793**	(NH4)6Mo7O24.4H2O	0,059
FE(NO3)3 9 H2O	2	0,00002	404,00	0,00004	**0,01616**	**0,808**	FeCl3. 6H2O	0,6381
Na2H2 2H2O	2	0,00002	372,24	0,00004	**0,0148896**	**0,74448**	NaEDTA	

FigureA3. Nutrient solution added to CWs, in order to maintain good nutritional conditions.

A4: Mobile phase

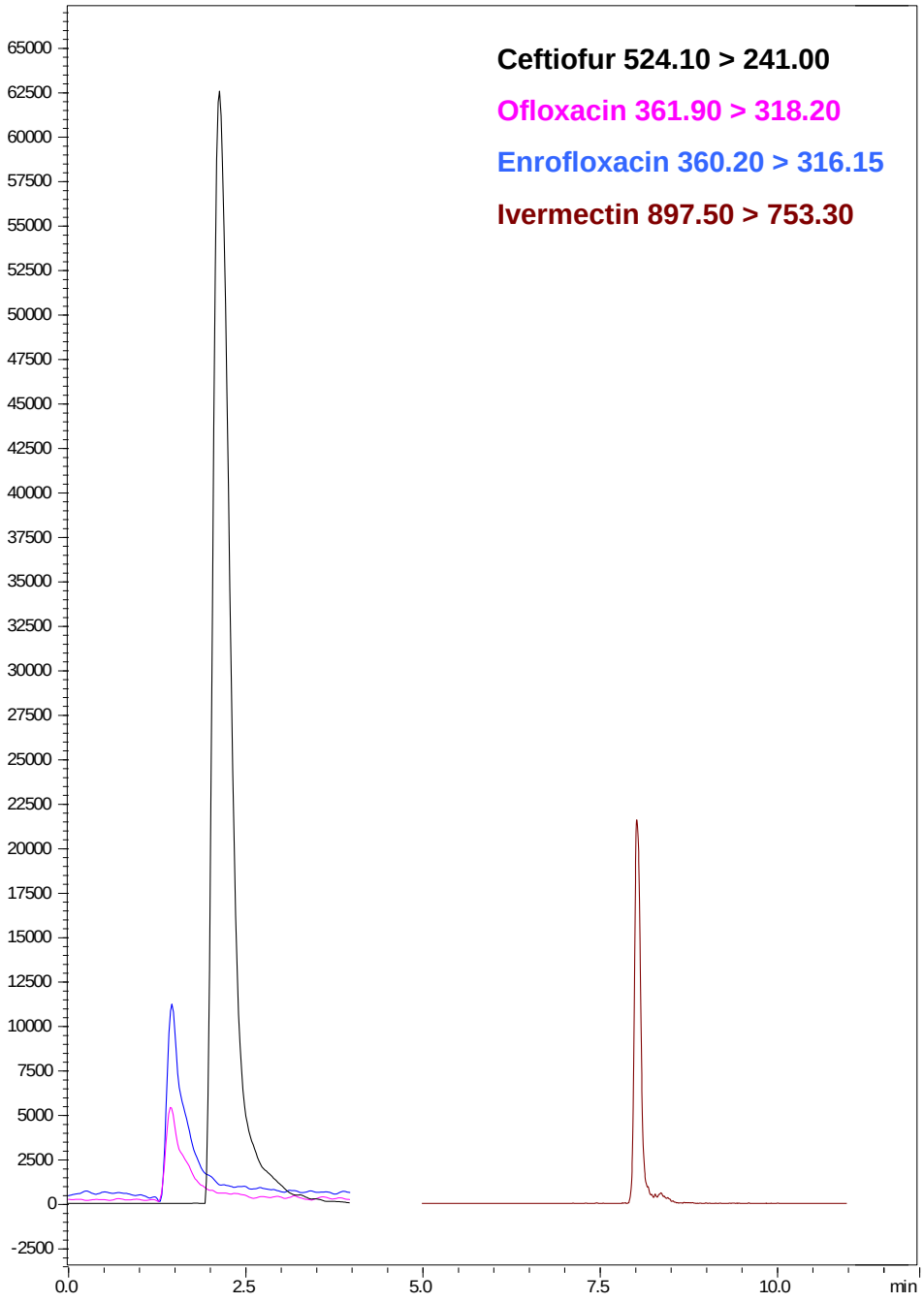


Figure A4. Chromatogram of the target analytes obtained with optimized mobile phase. Conditions: KinetexTM 1.7 µm XB-C18 100 Å column (100 × 2.1 mm, i.d.), using a mobile phase of methanol/water containing 0.1% formic acid performed at gradient mode at a flow rate of 0.20 mL min^{-1}.

A5: MS Parameters

- **Capillary voltage**

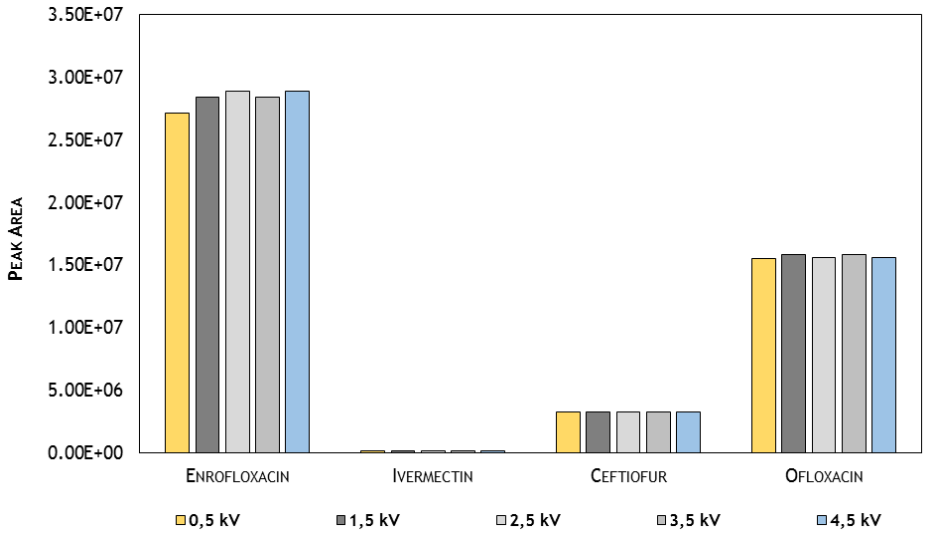


Figure A5a. Results obtained for veterinary pharmaceuticals with different capillary voltage values: 0.5, 1.5, 2.5, 3.5 and 4.5 kV.

- **Drying gas flow**

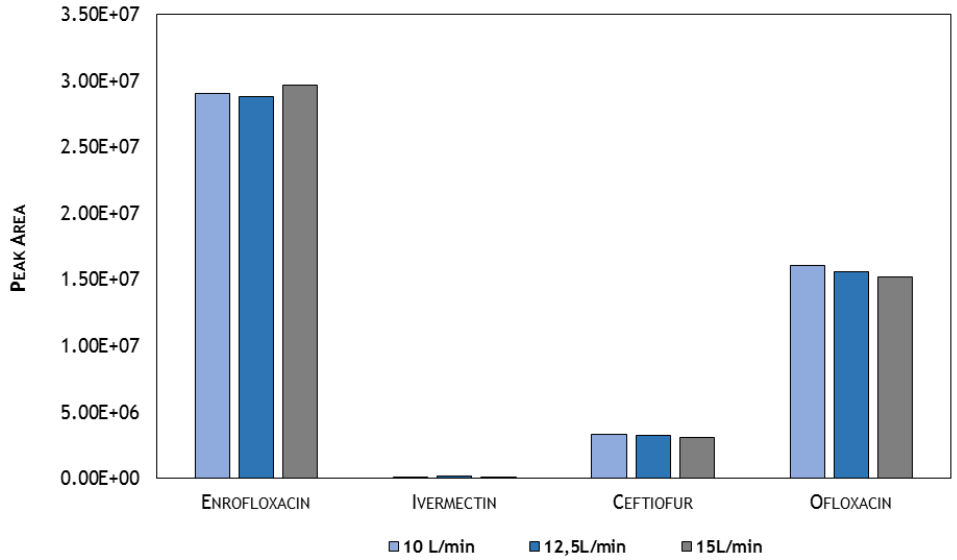


Figure A5b. Results obtained for veterinary pharmaceuticals with different drying gas flow values: 10, 12.5, and 15 dm^3 min^{-1}.

- **Nebulizing gas flow**

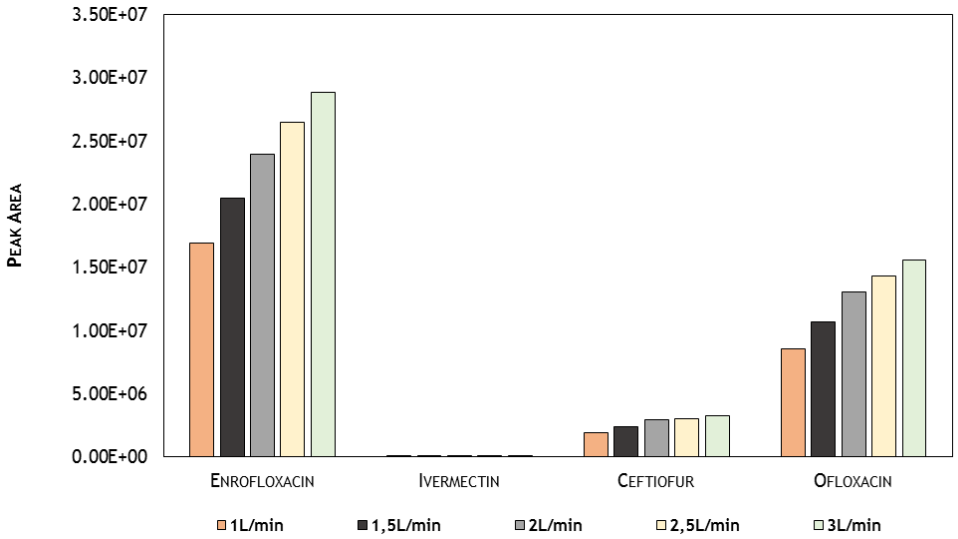


Figure A5c. Results obtained for veterinary pharmaceuticals with different nebulizing gas flow values: 1.0, 1.5, 2.0, 2.5 and 3.0 $dm^3\ min^{-1}$.

- **Desolvation temperature**

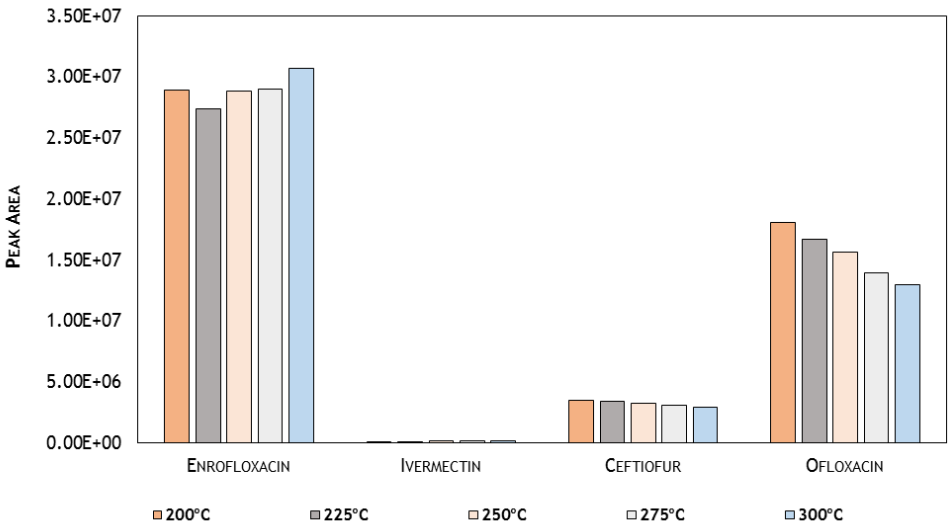


Figure A5d. Results obtained for veterinary pharmaceuticals with different desolvation temperature values: 200, 225, 250, 275 and 300 ºC.

- **Source temperature**

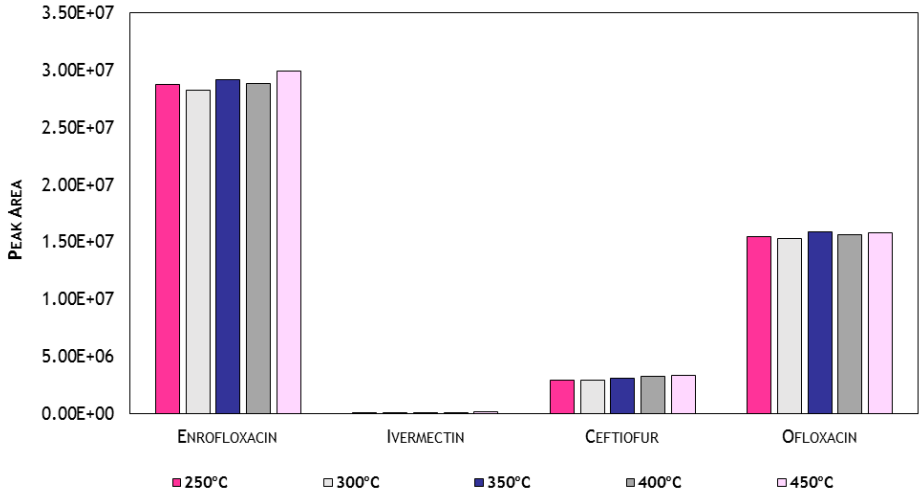


Figure A5e. Results obtained for veterinary pharmaceuticals with different source temperature values: 250, 300, 350, 400 and 450 ºC.

A6: Optimized mass spectrometer parameters for SRM analysis of Watch List compounds, Priority Substances and recalcitrant compounds

Table A6a. Optimized mass spectrometer parameters for SRM analysis of 11 CECs [166].

Group	Analyte	IS set[a]	ESI mode (NI[b] or PI[c])	Precursor ion (m/z)	Quantification (SRM1)				Confirmation (SRM2)				Ion ratio (± SD)
					Product Ion	DP[d] (V)	CE[e] (V)	CXP[f] (V)	Product Ion	DP[d] (V)	CE[e] (V)	CXP[f] (V)	
Anti-inflamrnatory	Diclofenac	1	NI	294.1	249.95	20	12	26	213.95	20	21	21	26.27 (± 0.17)
	Diclofenac-d4 (1)		NI	297.9	254.05	14	12	28	-	-	-	-	n.a.
Organic UV Filter	2-Ethylhexyl 4-methoxycinnamate	2	PI	291.2	179.1	-14	-9	-18	161.1	-14	-19	-15	1.01 (± 0.07)
Macrolide Antibiotics	Erythromycin	2	PI	734.4	158.15	-36	-34	-30	576.35	-36	-21	-28	2.28 (± 0.18)
	Clarithromycin	2	PI	748.4	158.15	-40	-30	-15	83.2	-40	-53	-30	3.06 (± 0.19)
	Azithromycin	2	PI	749.4	158.15	-38	-44	-28	83.15	-38	-54	-29	1.65 (± 0.13)
	Azithromycin-d3 (2)		PI	752.4	158.05	-38	-47	-14	-	-	-	-	n.a.
Pesticide	Methiocarb	3	PI	226.1	169.1	-24	-9	-17	121.1	-24	-19	-21	1.13 (± 0.16)
	Methiocarb-d3 (3)		PI	229.1	169.1	-25	-11	-30	-	-	-	-	n.a.
Neonicotinoids	Imidacloprid	4	PI	255.7	209.05	-30	-15	-21	175.05	-30	-18	-17	0.58 (± 0.31)
	Thiacloprid	4	PI	252.9	126	-28	-21	-21	99	-28	-44	-17	5.50 (± 0.17)
	Thiamethoxam	4	PI	291.9	211.1	-30	-14	-21	181.05	-30	-24	-17	2.98 (± 0.18)
	Clothianidin	5	PI	249.9	132	-29	-15	-23	169.05	-29	-13	-16	1.34 (± 0.15)
	Clothianidin-d3 (5)		PI	252.9	172	-29	-13	-17	-	-	-	-	n.a.
	Acetamiprid	4	PI	222.7	126	-15	-20	-23	56.1	-15	-16	-22	2.60 (± 0.19)
	Acetamiprid-d3 (4)		PI	226.1	126	-24	-21	-23	-	-	-	-	n.a.

[a] IS, internal standard; [b] NI, negative ionization mode; [c] PI, positive ionization mode; [d] DP, declustering potential; [e] CE, collision energy; [f] CXP, collision cell exit potential; [g] n.a, not applicable.

Table A6b. Optimized mass spectrometer parameters for SRM analysis of 4 PSs and 2 recalcitrant compounds [164].

Class and sub class	Analyte	IS set[a]	ESI mode (NI[b] or PI[c])	Precursor ion (m/z)	Quantification (SRM1)				Identification (SRM2)				Ion ratio (±SD)
					Product Ion (m/z)	DP[d] (V)	CE[e] (V)	CXP[f] (V)	Product Ion (m/z)	DP[d] (V)	CE[e] (V)	CXP[f] (V)	
Pharmaceuticals													
Macrolide Antibiotics	Azithromycin-d3(1)		PI	752.4	158.05	-38	-47	-14	-	-	-	-	n.a.
Psychiatric drugs	Carbamazepine	1	PI	236.9	194.10	-28	-20	-19	192.10	-28	-22	-19	4.22 (±0.18)
Pesticides													
Triazine	Atrazine[i]	2	PI	215.9	174.05	-23	-18	-30	68.15	-23	-37	-24	2.44 (±0.10)
	Simazine[i]	2	PI	201.9	124.10	-22	-18	-11	131.95	-22	-20	-23	1.35 (±0.20)
	Atrazine-d5 (2)		PI	221.0	179.05	-11	-19	-18	-	-	-	-	n.a.
	Isoproturon[i]	2	PI	206.9	72.10	-22	-21	-27	46.15	-22	-18	-16	2.19 (±0.07)
Herbicide	Clofibric acid	2	NI	213.1	127.00	10	13	13	85.00	10	11	13	8.42 (±0.31)
Industrial compound	Perfluorooctanesulfonic acid[i]	2	NI	498.7	79.95	18	50	14	99.00	18	46	18	3.15 (±0.13)

[a] IS is internal standard.
[b] NI is negative ionization mode.
[c] PI is positive ionization mode.
[d] DP is the declustering potential.
[e] CE is the collision energy.
[f] CXP is the collision cell exit potential.
[g] n.a. is not applicable.
[h] Included in the watch list for the intent prioritization process at European Union level (Annex of the EU Decision 2015/495).
[i] PSs of the Directive 2013/39/EU.

A7: Validation parameters for Watch List compounds.

Table A7a. Retention time, range, linearity, instrument and method detection and quantification limits for 11 CECs [166].

Group	Analyte	Retention time (min)	Range (ng L^{-1})	r^2	IDL[a] (µg L^{-1})	IQL[b] (µg L^{-1})	MDL[c] (ng L^{-1})	MQL[d] (ng L^{-1})
Anti-inflammatory	Diclofenac	3.33	0.90–300	0.997	0.59	1.80	0.30	0.90
Organic UV Filter	2-Ethylhexyl 4-methoxycinnamate	4.28	8.08–100	0.995	5.33	16.16	2.67	8.08
Macrolide Antibiotics	Erythromycin	4.60	0.51–300	0.996	0.34	1.02	0.17	0.51
	Clarithromycin	5.25	0.03–100	0.994	0.02	0.07	0.01	0.03
	Azithromycin	5.30	0.37–100	0.997	0.24	0.73	0.12	0.37
Pesticide	Methiocarb	2.51	0.54–100	0.997	0.35	1.07	0.18	0.54
Neonicotinoids	Imidacloprid	1.72	6.06–400	0.996	4.00	12.12	2.00	6.06
	Thiacloprid	1.72	0.08–400	0.999	0.05	0.16	0.03	0.08
	Thiamethoxam	1.65	3.71–400	0.998	2.45	7.42	1.22	3.71
	Clothianidin	1.71	0.73–400	0.998	0.48	1.47	0.24	0.73
	Acetamiprid	1.69	0.98–75	0.996	0.64	1.95	0.32	0.98

[a] IDL, instrument detection limit; [b] IQL, instrument quantification limit.; [c] MDL, method detection limit; [d] MQL, method quantification limit.

Table A7b. Recovery, accuracy and precision (intra- and inter-batch) for 11 CECs [166].

Group	Analyte	Recovery (%)	Accuracy (%)	Intra-batch precision RSD (%)	Inter-batch precision RSD (%)
Anti-inflammatory	Diclofenac	103.93 ± 8.70	87.19–115.91	< 7.08	< 12.12
Organic UV Filter	2-Ethylhexyl 4-methoxycinnamate	20.42 ± 6.17	91.47–118.99	< 6.27	< 7.63
Macrolide Antibiotics	Erythromycin	4.53 ± 0.16	95.40–114.74	< 16.68	< 17.83
	Clarithromycin	105.62 ± 5.43	80.61–111.55	< 17.66	< 16.91
	Azithromycin	126.08 ± 18.03	80.09–117.04	< 10.16	< 7.11
Pesticide	Methiocarb	148.64 ± 6.47	83.81–115.08	< 8.79	< 8.81
Neonicotinoids	Imidacloprid	40.91 ± 13.18	84.72–109.43	< 16.68	< 12.46
	Thiacloprid	47.45 ± 11.50	88.47–90.47	< 11.16	< 11.46
	Thiamethoxam	25.90 ± 6.83	85.14–93.42	< 7.74	< 8.92
	Clothianidin	27.54 ± 9.42	87.48–87.79	< 9.45	< 8.23
	Acetamiprid	52.44 ± 7.77	99.29–116.10	< 10.87	< 9.22

[a] IDL, instrument detection limit; [b] IQL, instrument quantification limit.; [c] MDL, method detection limit; [d] MQL, method quantification limit.